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The role of platelets in the pathology of cancer.

Karl Egan
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Signed Karl Egan

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Date 4/1/2013

THE ROLE OF PLATELETS IN THE PATHOLOGY OF CANCER

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Published papers during PhD

(1) Platelet Adhesion and Degranulation Induce Pro-Survival and Pro-Angiogenic Signalling in Ovarian Cancer Cells. **Egan K**, Crowley D, Smyth P, O'Toole S, Spillane C, Martin C, Gallagher M, Canney A, Norris L, Conlon N, McEvoy L, Ffrench B, Stordal B, Keegan H, Finn S, McEneaney V, Laios A, Ducrée J, Dunne E, Smith L, Berndt M, Sheils O, Kenny D, O'Leary J. *PLoS One.* 2011; **6(10):**e26125.

(2) Reducing intra-individual variation in platelet aggregation: implications for platelet function testing. Peace AJ, Egan K, Kavanagh GF, Tedesco AF, Foley DP, Dicker P, Berndt MC, Kenny D. J Thromb Haemost. 2009 Nov; 7(11):1941-3.

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(3) Increased platelet reactivity in patients with late-stage metastatic cancer. *Cooke NM, *Egan K, McFadden S, Grogan L, Breathnach OS, Hennessy BT, Kenny, D. Manuscript submitted to Cancer Research in October 2012 for publication as a short communication. * Joint first authors

Abbreviations

ACD - Acid Citrate Dextrose

ADP - Adenosine Diphosphate

ATP - Adenosine Triphosphate

BSA - Bovine serum albumin

CAPRIE – Clopidogrel vs. Aspirin in Patients at Risk of Ischemic Events trial

CHARISMA - Clopidogrel for High Atherothrombotic Risk and Ischemic Stabilisation, Management, and Avoidance trial

COX-1 - cyclooxygenase -1

COX-2 - cyclooxygenase -2

CRP - C-reactive protein

DMEM - Dulbecco's Modified Eagle's Medium

DNA - deoxyribonucleic acid

DVT – Deep Vein Thrombosis

ECM - Extracellular matrix

EDTA - ethylenediaminetetraacetic acid

FBS - Foetal Bovine Serum

GPS - Gray Platelet Syndrome

LDH - Lactate Dehydrogenase

MFI - Mean fluorescence intensity

mRNA - messenger ribonucleic acid

NCHCD - National Centre for Heritable Coagulation Disorders

NK cell - Natural Killer cell

PAR-1 - Protease activated receptor 1

PAR-4 - Protease activated receptor 4

PDGF - platelet derived growth factor

PGH2 synthase - Prostaglandin H2 synthase

PLATO - Platelet Inhibition and Patient Outcomes trial

PLTs - Platelets

PPP - Platelet poor plasma

PRP - Platelet rich plasma

RCSI - Royal College of Surgeons in Ireland

RNA - ribonucleic acid

RPMI - Roswell Park Memorial Institute

SCID – Severe combined immunodeficiency

SPA - Spontaneous platelet aggregation

TGFß - Transforming growth factor β

TF – Tissue Factor

TFPI – Tissue factor pathway inhibitor

TxA2 – Thromboxane A2

TRITON-TIMI 38 - Therapeutic Outcomes by Optimising Platelet Inhibition by Prasugrel-Thrombolysis in Myocardial Infarction 38 Trial

VEGF - vascular endothelial growth factor

VWF - Von Willebrand Factor

SUMMARY

Beyond their role in haemostasis, platelets are extremely important in the pathology of cancer. They are implicated in the promotion of growth and angiogenesis in the primary tumour, maintaining the integrity of tumour associated vasculature, and facilitating metastasis. In this thesis, novel mechanisms by which platelet function can influence malignancy and how in turn, malignancy can influence platelet function are demonstrated. Firstly, platelet adhesion and degranulation were shown to induce proliferative, anti-apoptotic, and pro-angiogenic signalling in ovarian cancer cells that could influence the metastatic potential of ovarian cancer cells *in vivo*. Secondly, platelets were shown to be capable of protecting cancer cells from shear stress induced damage. This could promote their survival within the circulation during bloodborne metastasis. Thirdly, patients with metastatic cancer were shown to display global platelet hyperreactivity, a phenomenon that could contribute to the high incidence of cancer associated thrombosis.

CHAPTER 1: INTRODUCTION

1.1 General Introduction

Metastasis is responsible for 90 % of cancer related deaths in patients with solid tumours. It is a complex, multistep process involving the spread of malignant cells from the primary tumour site to distant organs via the lymphatic system or blood circulation[1]. During bloodborne metastasis, tumour cells must survive the negative pressures of the circulation system (immune surveillance and hemodynamic forces), before migrating out of blood vessels through the vascular endothelium, to eventually proliferate at a distant site [1, 2]. During hematogenous dissemination, the ability of tumour cells to interact with (and activate) endothelial cells (*section 1.3*), coagulation proteins *(Section 1.4)*, and platelets *(Section 1.5)* is believed to promote their survival within the circulation and therefore, facilitate metastasis. Endothelial cells, coagulation proteins, and platelets are the principle components of the hemostatic system *(section 1.2)*, and are responsible for maintaining the integrity of the vascular system. They function to prevent excessive blood loss at sites of vascular damage through the formation of stable platelet - fibrin rich clots[3]. In this introduction, the evidence implicating the haemostatic system in metastasis will be presented, focusing predominantly on the role of platelets in metastasis, the topic of this thesis.

1.2 Haemostasis: Endothelium, platelets and the coagulation cascade

The haemostatic system acts to prevent excessive blood loss through the formation of a platelet and fibrin rich clots at the site of vascular damage. Under normal conditions, the endothelium prevents clot formation by acting as a physical barrier between the thrombogenic subendothelial matrix and flowing blood, as well as through the release of soluble factors that inhibit platelet function (prostacyclin, nitric oxide, and CD39-ectoADPase pathways) and the action of clotting factors (thrombomodulin) [4, 5]. The endothelium consists of a single layer of endothelial cells that covers the interior wall of all blood vessels, from the heart to the smallest capillary. Upon damage to this layer, the thrombogenic subendothelial matrix is exposed to flowing blood, initiating haemostasis[4, 5]. Haemostasis proceeds via platelet mediated primary haemostasis and the coagulation cascade mediated secondary haemostasis. Both primary and secondary haemostasis occur simultaneously and act synergistically to promote clot formation[6].

Primary Haemostasis: Due to margination induced by red blood cells, platelets circulate in close proximity to the vessel wall, placing them in an ideal position to survey vascular integrity. Following vascular damage, platelets rapidly adhere to exposed extracellular matrix (ECM) proteins, become activated, then aggregate to form a platelet plug. It is an extremely complex, tightly regulated process involving a growing list of platelet receptors and signalling molecules[3]. Firstly, collagen in the subendothelial ECM is exposed, allowing von Willebrand factor (VWF) to complex with collagen fibrils[7, 8]. Under high shear conditions, platelets tether to the exposed ECM via GPIb-IX-V mediated adhesion to VWF. Following tethering, GPVI and integrin $\alpha 2\beta 1$ interactions with collagen allow stable platelet adhesion[9]. These initial events trigger intracellular signalling within platelets[10]. The adhered platelets spread and release soluble agonists, while platelet integrin $\alpha IIb\beta 3$ undergoes a conformational change to its active state.

During activation induced cytoskeletal rearrangements, discoid platelets extend filopodia and lamellopodia, flatten out and adhere more closely to the site of vascular damage. ADP *(stored in platelet dense granules)* and Thromboxane A_2 *(synthesised from arachidonic acid via prostaglandin biosynthesis)* are released from activated platelets. Both cause the activation and recruitment of more platelets to the growing thrombus, thereby creating a positive feedback mechanism that promotes the haemostatic response. Platelets express at least two receptors for ADP, the G protein coupled purinergic receptors P2Y1 and P2Y12[11, 12]. TxA2 acts through the G protein coupled TxA_2 (TP) receptor[13]. The importance of these two pathways is highlighted by the fact they are the targets for the most widely used antiplatelet agents, aspirin (inhibits prostaglandin synthesis) and P2Y12 receptor antagonists (clopidogrel, prasugrel, ticagrelor). Activated, but not resting integrin αIIbβ3 can bind its ligands fibrinogen and vWF in suspension[14]. Fibrinogen mediated bridging of platelets via activated integrin αIIbβ3 facilities platelet aggregation[3] leading to the formation of the initial platelet plug or thrombus (Figure 1.1).

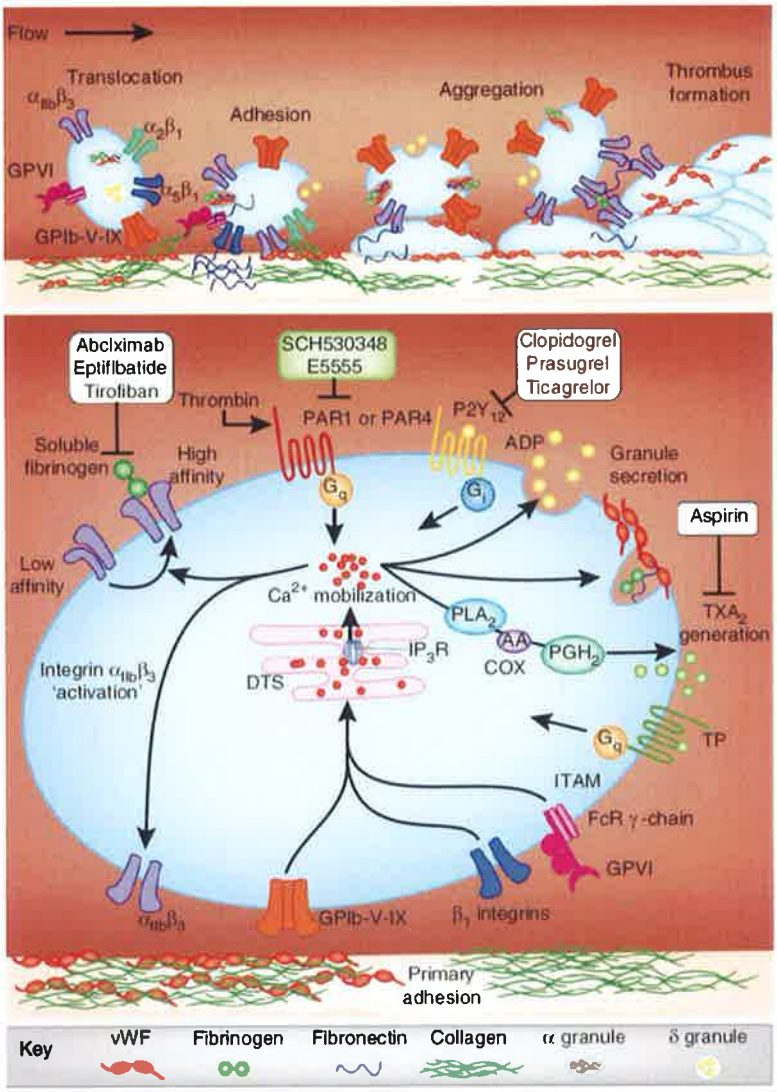

Figure 1.1: Schematic representation of the events involved in primary haemostasis. Following vessel damage, collagen in the subendothelial extracellular matrix is exposed, allowing von Willebrand factor (VWF) to complex with collagen fibril. Under high shear, the initial tethering of platelets to the exposed ECM is via GPIb-IX-V mediated adhesion to vWF. Following tethering, GPVI and integrin α2β1 interactions with collagen allow stable platelet adhesion. These initial events trigger intracellular signalling within platelets. The adhered platelets spread, release soluble agonists, and integrin αIIbβ3 undergoes a conformational change to its active state. The release of soluble agonists promotes the haemostatic response, activating and recruiting more platelets to the growing thrombus. Fibrinogen mediated bridging of adjacent platelets via activated αIIbβ3 facilities platelet aggregation. TxA2 synthesis, P2Y12, αIIbβ3, and PAR-1 are common molecular targets for antiplatelet agents. Aspirin inhibits prostaglandin synthesis, and therefore the production and release of TxA2. Clopidogrel, prasugrel, and ticagrelor inhibit the P2Y12 receptor. Abciximab, tirofiban, and eptifibatide inhibit integrin αIIbβ3, while SCH530348 and E5555 inhibit the PAR-1 receptor. Adapted from Jackson (2011)[15].

Secondary Haemostasis: The initial platelet plug is eventually stabilised by the formation of an insoluble fibrin meshwork via the action of the coagulation cascade. The coagulation cascade is a series of proteolytic reactions that convert inactive precursors into active coagulation factors, ultimately leading to fibrin formation. The cascade has two separate initial pathways, the contact activation dependent pathway and the tissue factor dependent pathway. The contact activation dependent pathway is initiated when blood comes in contact with negatively charged surfaces, such as ECM matrix collagen. The tissue factor dependent pathway is initiated in response to deep tissue injury. Tissue factor (TF) is expressed on the membrane of extravascular cells. Upon exposure to blood, TF binds coagulation factor VII and its active form FVII. Both pathways lead to the activation of Factor X (FXa). Of the two initial pathways, the tissue factor pathway is the most potent, resulting in the rapid production FXa[16-18].

The two pathways eventually converge in a final common pathway, the generation of thrombin. FXa is the first coagulation protein of this common pathway. It forms a Ca^{2+} dependent complex with factor Va on the surface of a cellular phospholipid membrane, provided by the surface of activated platelets. This complex, the prothrombinase complex, converts inactive prothrombin to thrombin. Thrombin is a multifunctional serine protease that converts soluble fibrinogen to insoluble fibrin through the cleavage and release of fibrinopeptide A and fibrinopeptide B. The local generation of thrombin also promotes platelet activation and aggregation through the proteolytic cleavage of the G protein coupled protease activated receptors (PAR-1 & PAR-4)[19]. Fibrin molecules polymerise and are cross linked (via FVIIIa activity) to form an insoluble fibrin meshwork that stabilises the initial platelet plug. In the cascade, the anticoagulant properties of tissue factor pathway inhibitor (TFPI), thrombomodulin, antithrombin, and activated protein C serve as regulatory mechanisms preventing uncontrolled thrombin generation [16-18] (Figure 1.2).

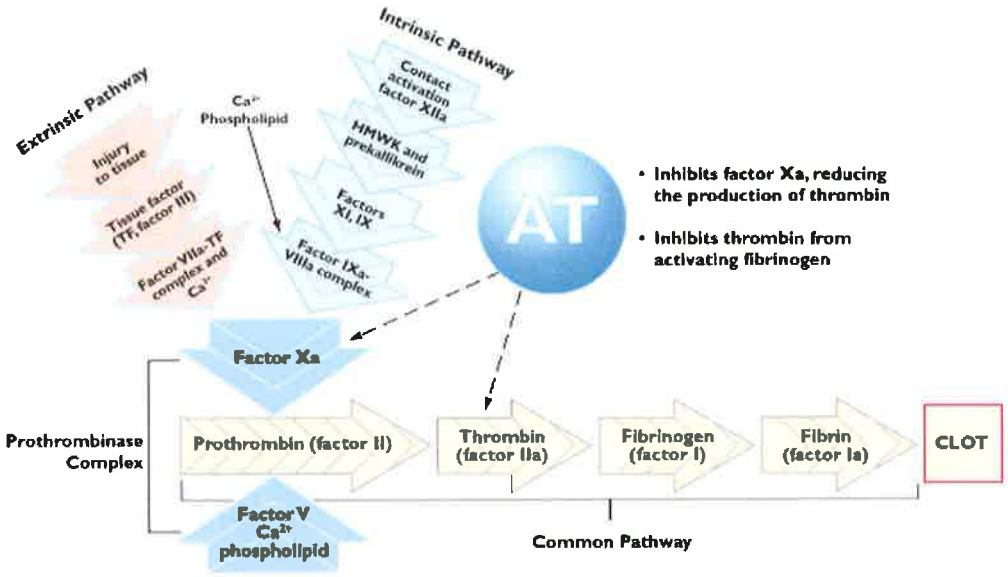

Figure 1.2: Schematic representation of secondary haemostasis, the coagulation cascade. This coagulation cascade is a series of enzymatic reactions that convert inactive precursors into active coagulation factors, ultimately leading to fibrin formation. The cascade has two separate initial pathways, the contact activation pathway and the tissue factor pathway that provide alternative routes for the generation of activated factor X. These two pathways converge in a final common pathway, the generation of thrombin and the thrombin dependent cleavage of fibrongen to fibrin. Adapted from Escobar et al[20].

1.3 The haemostatic system and metastasis

Beyond their role in haemostatic system, endothelial cells, coagulation proteins, and platelets play an important role in metastasis. This is convincingly shown in both experimental and spontaneous metastasis models. In experimental metastasis models, tumour cells are injected directly into the circulation and the development of metastaic foci is quantified. This assay is advantageous given the shortened time to development of metastatic foci, but its physiological significance is questionable given that it bypasses the early stages of the metastatic cascade. Conversely, spontaneous metastasis models assess the whole process, from proliferation at the primary tumour site to subsequent metastasis, and hence are more physiologically relevant [21]. Commonly, the development of metastatic lesions in the lung is used to quantify metastasis in these models. From this, it appears that circulating tumour cells arrest in the pulmonary vasculature through mechanical restriction in small blood vessels or via direct adhesive interactions with endothelial cells[22]. This event triggers coagulation and platelet activation, leading to the formation of a platelet – fibrin rich clot. This protective clot prolongs the survival of arrested tumour cells in the circulation and facilitates extravasation out of the vasculature [23, 24]. Genetic defects in, or pharmacological inhibition of endothelial cell, coagulation protein, and platelet function are associated with decreased metastasis.

1.3.1 Endothelium and metastasis

The endothelium consists of a single layer of endothelial cells covering the interior wall of all blood vessels, from the heart to the smallest capillary. The ability of tumour cells to adhere to endothelial cells is important for vascular arrest during circulation[22, 25]. Various studies have shown direct tumour cell adhesion to endothelial cells *in vivo* mediated by endothelial cell adhesion molecules, including members of the selectin family [22, 26, 27]. The members of the selectin family of adhesion molecules share a common ability to recognise the tetrasaccharide sialyl Lewis x motif, commonly found on the terminal end of glycans attached to glycoproteins or glycolipids. Tumour cells, particularly adenocarcinoma cell lines, are known to express sialyl Lewis x type ligands that are recognised by selectins [28-30].

Interestingly, increased expression of sialyl Lewis x in tumours is associated with poorer prognosis in patients due to increased metastasis [31-33]. Endothelial cells express E-selectin in response to inflammation. Its expression also increases following the arrest of tumour cells in the vasculature of the lung [34, 35]. In a murine model of experimental metastasis, the ability of colon cancer cells to arrest in the lung of cytokine treated mice was dependent on endothelial E-selectin expression. Following treatment with a soluble E-selectin fusion protein, the arrest of colon cancer cells in the lung and subsequent development of metastatic lesions was dramatically decreased[36]. Interestingly, overexpression of E-selectin in the liver of mice increases metastaic seeding of colon cancer cells to this site [37].

The importance of endothelial cell adhesion molecules in bloodborne dissemination is also demonstrated in studies on the effect of activated Protein C on metastasis. Activated Protein C, an anticoagulant *in vivo,* can interact directly with endothelial cells through its counter receptor, endothelial Protein C receptor (EPCR) [38]. This interaction has been shown to decrease the expression of adhesion molecules on endothelial cells, some of which play an important role in metastasis. The overexpression of EPCR in endothelial cells in mice is associated with a significant reduction in experimental metastasis to both the liver and lung. Similarly, mice treated with recombinant activated Protein C also display decreased experimental metastasis. This reduction in metastasis was associated with a decrease in endothelial cell levels of adhesive molecules, for example, P-selectin [38].

1.3.2 Coagulation and metastasis

As mentioned previously, the coagulation cascade is a series of proteolytic reactions that convert inactive precursors into active coagulation factors. This series of reactions leads to thrombin generation, and subsequently fibrin formation. The two most important molecules in the coagulation cascade are tissue factor and thrombin, both of which have been shown to have a critical role in metastasis, as well as haemostasis.

Tissue factor: Tissue factor (TF) is a 47kDa transmembrane protein expressed on the membrane of most extravascular cells. Upon exposure to blood, TF binds coagulation factor VII and its active form FVII, leading to the activation of factor IX and X. The activation of Factor X drives thrombin generation and fibrin formation. Numerous tumour cell lines have been shown to express tissue factor, giving them intrinsic procoagulant properties[17]. Bromberg et al produced 2 variants of the B16 melanoma cell line, one with high TF expression, one with low TF expression. In a model of experimental pulmonary metastasis, metastaic lesions were observed in 86 % of mice injected with high TF expressing B16 melanoma cells, but in only 14 % of mice injected with low TF expressing B16 melanoma cells, suggesting TF promotes metastasis. TF expression was not associated with increased vascular arrest of melanoma cells; rather it prolonged melanoma cell survival within the pulmonary vasculature[39].

Monoclonal antibodies against TF inhibit melanoma metastasis in a murine model of experimental metastasis[40]. In humans, TF expression correlates with stage of malignancy in glioma patients[41]. Its expression is increased in metastatic breast cancer cells compared to non metastaic breast cancer cells [42]. TF expression is also an independent risk factor for hepatic metastasis in patients with colon cancer [43]. Consistent with the pro-metastaic effect of TF, intravenous injection of recombinant murine tissue factor pathway inhibitor *(a small polypeptide that inhibits Factor Xa, thrombin, and the Factor VIIa-tissue factor complex)* is associated with a significant reduction in experimental melanoma pulmonary metastasis. Furthermore, B16

melanoma cells transfected with a tissue factor pathway inhibitor expression vector display decreased experimental metastasis compared to wild type B16 melanoma cells [44].

Thrombin: Thrombin is a multifunctional serine protease with a significant role in haemostasis. It can activate platelets via PAR receptors, convert fibrinogen to fibrin, and activate endothelial cells (VWF release, P-selectin expression) [23]. Thrombin treated colon and melanoma tumour cells show a 10 to 156 fold increase in experimental metastasis [45]. The increased expression of the thrombin receptor PAR-1 on B16 melanoma cells increases experimental metastasis up to 5 fold [46]. Mice deficient in the thrombin receptor PAR-2 display reduced tumour growth and are protected against spontaneous metastasis in a model of breast cancer [47]. Hirudin, (a thrombin inhibitor) is associated with decreased tumour growth, tumour cell seeding into the bloodstream, decreased spontaneous metastasis to the lung, and death in mice with highly aggressive 4T1 breast cancer tumours [48]. Argatroban, another thrombin inhibitor, has also been shown to inhibit the experimental bone metastasis of both melanoma and breast cancer cell lines [49, 50].

As well as having procoagulant properties, thrombin also has anticoagulant properties. Endothelial cell membrane bound thrombomodulin forms a complex with thrombin causing it to undergo functional transformation from a pro-coagulant to an anti-coagulant. This thrombin-thrombomodulin complex activates Protein C, leading to the inactivation of Factor Va and VIIa and the attenuation of thrombin generation. Mice expressing a mutant form of thrombomodulin with decreased affinity for thrombin are characterised by significantly increased B16 melanoma and Lewis Lung carcinoma cell experimental metastasis compared to wild type mice. This suggests thrombomodulin has anti-metastaic properties *in vivo* and confirms the pro-metastaic effect of thrombin[51]. In patients, thrombomodulin expression correlates with metastasis in patients with oesophageal squamous cell carcinoma [52].

1.4 Platelets

As previously described, the primary role of platelets is in haemostasis (Section 1.2). Following vascular damage, they rapidly adhere to exposed ECM proteins, become activated, then aggregate to form a platelet plug. However, beyond their primary role in haemostasis, platelets are now seen as multifunctional cells with a role in a variety of physiological and pathophysiological processes. It has become increasingly apparent that platelets play an important role in the progression and pathology of cancer. Experimental animal models suggest platelets play a key role in facilitating bloodborne metastasis *(Section 1.4.2)* and maintaining the integrity of tumour vasculature *(Section 1.4.4)*. The antiplatelet agent aspirin has also been shown to decrease the incidence of cancer in humans *(Section 1.4.5)*.

1.4.1 General Platelet Biology

Platelets are anuclear blood cells that circulate for approximately 10 days at levels between 150 - 400 $\times 10^9$/l, making them the second most abundant blood cell after erythrocytes. Platelets are relatively simple cells, they lack a nucleus but are characterised by a vast, complex membrane system and cell specific organelles. They are surrounded by a phospholipid plasma membrane. The plasma membrane connects with the open canalicular system (OCS), an internal membrane system, to greatly increase the surface area of the platelet. This OCS facilitates the uptake of non-megakaryocyte derived proteins and the secretion of the contents of platelet storage granules. Platelets have a second internal membrane system called the dense tubular system, the platelet equivalent of the smooth endoplasmic reticulum. It is the site where enzymes involved in prostaglandin synthesis are localised, and the major site of calcium uptake [53].

Platelets contain a wide array of organelles, including mitochondria, glycogen granules, peroxisomes and lysosomes. The storage granules (α and dense) are specific to platelets. Upon platelet activation, these storage granules fuse with the plasma membrane and release their content. Coppinger et al [54] have identified >

350 proteins that are released from platelet storage granules following activation. α- Granules are the most abundant platelet organelle, with 40 - 80 per platelet. They are oval shaped, and approximately 200 - 500 nm in diameter. α- granules contain a wide array of proteins, those synthesised in the megakaryocyte as well as those taken up via receptor mediated endocytosis. These proteins include platelet factor 4, fibrinogen, fibronectin, von Willebrand factor, thrombospondin, β-thromboglobulin, and platelet derived growth factor. Dense granules are smaller than α –granules and found in smaller numbers, 4-8 per platelet. ATP (Adenosine Triphosphate), ADP (Adenosine Diphosphate), serotonin, pyrophosphate, magnesium and calcium are all found at high concentrations within dense granules [53].

Platelets are derived form megakaryocytes, a highly specialized precursor cell found in bone marrow. The development of megakaryocytes is characterised by a unique process called endomitosis. They undergo multiple rounds of DNA replication, but in the absence of cell division, as mitosis is prematurely terminated. This results in the formation of a giant polyploidal cell. As such, megakaryocytes have an increased number of diploid chromosomes (4N, 16N, 32N or 64N). After endomitosis, the cytoplasm fills with organelles and proteins specific to platelets. Eventually, the megakaryocyte undergoes a massive reorganisation, with the formation of long cytoplasmic extensions characterised by multiple platelet swellings linked by cytoplasmic bridges. The rapid retraction of the cytoplasmic bridges separates the platelets from the main megakaryocyte body and they are released into the circulation. The development of megakaryocytes and the formation of platelets is controlled predominantly by the hormone thrombopoietin. Both megakaryocytes and platelets express a surface membrane bound receptor (c-mpl) that binds thrombopoietin. This interaction causes a wide variety of effects, including the prevention of apoptosis (programmed cell death), an increase in the size, number and ploidy of megakaryocytes, and increased megakaryocyte maturation [55].

1.4.2 Platelets and Metastasis

Of the 3 elements of the haemostatic system, platelets were first shown to promote metastasis. They have been shown to support the bloodborne dissemination of a range of tumour cell lines in models of both spontaneous and experimental pulmonary metastasis. In 1968, Gasic *et al* first demonstrated that pharmacologically inducing thrombocytopenia caused a reduction in the development of metastatic foci by TA_3 ascites tumour cells in a CAF_1/JAX murine model of pulmonary metastasis. This anti-metastatic effect could be reversed by transfusing platelets into the thrombocytopenic mice[56]. Genetically induced thrombocytopenia is also associated with reduced metastasis. Camerer *et al* showed the development of B16 melanoma metastatic foci was reduced by 96 % in NF-E2$^{-/-}$ mice compared to wild type mice in both syngenic/immunocompetent (C57BL6) and SCID (severe combined immunodeficiency) mice. NF-E2 is a transcription factor required for platelet production, independent of thrombopoietin and megakaryocyte growth and differentiation factor levels. NF-E2$^{-/-}$ mice lack circulating platelets and their megakaryocytes display no cytoplasmic platelet formation [57].

The role of platelet receptors or platelet signalling molecules in metastasis has also been examined. These studies have used pharmacological inhibition or knockout mouse models. They have served to highlight the complexity and heterogeneity of interactions between cancer cells and platelets, since deficiencies in nearly all platelet receptors or signalling molecules are associated with reduced metastasis. For example, in a mouse model of experimental metastasis, mice deficient in the thrombin receptor PAR-4[58], the G protein Gaq [59], and the collagen receptor GPVI [60] all show abnormalities in platelet function (decreased activation or aggregation) that confer protection against experimental melanoma pulmonary metastasis. As well as protecting against experimental metastasis, a deficiency in Gaq (a G-protein required for platelet activation in response to multiple agonists) also protects against spontaneous metastasis [59]. Pharmacological inhibitors of integrin αIIbβ3 also decrease melanoma metastasis [61]. Integrin αIIbβ3 is the most abundant

receptor on the platelet surface, with approximately 50-80000 per platelet. It facilitates platelet aggregation by cross linking platelets via its major ligand fibrinogen[14]. Consistent with the anti-metastatic effect of integrin αIIbβ3 and thrombin antagonists, fibrinogen$^{-/-}$ mice are also protected against experimental melanoma metastasis. As well as being protective in experimental metastasis models, fibrinogen$^{-/-}$ mice are also protected against spontaneous metastasis [62]. Ulckan *et al* have shown that aspirin and ATP102 (an ADPase) reduce melanoma metastasis in a murine model of bone metastasis, also indicating a role platelet TxA2 (TP) receptor and P2Y1/P2Y12 receptor signalling in metastasis [63].

Kim *et al* showed decreased pulmonary metastasis of LS180 and T-84 human colon cancer cells in P-selectin$^{-/-}$ Rag2$^{-/-}$mice[64]. P-selectin is present in platelet α granules and the Weibel Palade bodies of endothelial cells. Upon platelet activation, it translocates to the platelet membrane surface and serves to mediate platelet adhesive interactions with monocytes via its major ligand PSGL-1 (P-selectin glycoprotein ligand 1)[65]. In this study, they demonstrated three potential mechanisms of protection against metastasis. Intravenously injected colon cancer cells homed to the lungs at a slower rate in P-selectin$^{-/-}$ mice, platelets from P-selectin$^{-/-}$ mice did not adhere to colon cancer cells, and platelet clumping around colon cancer cells in the lung was markedly decreased in P-selectin$^{-/-}$ mice. Coupland *et al* have also shown that P-selectin$^{-/-}$ mice are protected against spontaneous metastasis. Given that endothelial cells also express P-selectin upon activation, it is possible that the deficiency in endothelial P-selectin is partly responsible for the observed anti-metastaic effect. Using mice specifically deficient in either platelet or endothelial P-selectin, Coupland et al showed that mice deficient in either endothelial or platelet P-selectin are protected against metastasis, demonstrating the importance of both sources of P-selectin[66].

While defects in the majority of platelet receptors have been associated with reduced metastasis, this is not the case for all platelet receptors. In the case of GPIbα, conflicting results have been reported by different groups. For example, pharmacological inhibition of platelet GPIbα with monoclonal antibodies is

associated with increased metastasis, suggesting that this receptor has anti-metastatic properties[67]. This data is conflicting with results from genetic studies. GPIbα-/- mice are protected from metastasis, suggesting that this receptor has pro-metastatic properties[68]. This discrepancy could be explained by the biological properties of GPIbα. It is an extremely important receptor in platelet biology, not only mediating platelet adhesion via its major ligand, VWF, but it is also required for maintaining membrane integrity during megakaryocyte maturation. GPIbα$^{-/-}$ mice have a bleeding phenotype characterised by giant platelets and a reduced platelet count, similar to the phenotype of Bernard Soulier syndrome in humans[69]. In contrast, pharmacological inhibition of GPIbα would not affect platelet size, count, or development[70].

This story is further complicated by studies on the role of the GPIbα ligand VWF in metastasis. VWF is a multimeric plasma glycoprotein, synthesised by both megakaryocytes and endothelial cells. Platelet VWF is stored in alpha granules; while endothelial cell derived VWF is stored in Weibel Palade bodies or released into plasma. Pharmacological inhibition of plasma VWF is associated with reduced metastasis[71], whereas a total VWF deficiency in mice is associated with increased metastasis[72]. However, VWF$^{-/-}$ mice show multiple abnormalities. In the absence of VWF, Weibel Palade storage granules in endothelial cells do not form, meaning there is an altered expression of multiple proteins, including P-selectin, in endothelial cells in these mice. Yet, Terraube *et al* showed that restoration of plasma vWF corrected the pro-metastatic effect associated with VWF deficiency, suggesting that their results were not due to the altered trafficking of other proteins in endothelial cells[72].

1.4.3 Platelet Cloaking: Platelet mediated protection of tumour cells from immune surveillance.

Since it has become apparent that platelets facilitate metastasis, investigators have started to focus on the mechanism underlying platelet mediated metastasis. Studies have shown that platelets rapidly adhere to tumour cells *in vitro* and tumour-platelet emboli have been observed *in vivo* [64, 71, 73-80]. This phenomenon of platelet-tumour cell adhesion has been termed 'platelet cloaking' owing to the protective effect platelet adhesion has been shown to have on tumour cells [65, 81]. Platelet cloaking appears to facilitate successful metastasis by protecting cancer cells from immunological assault, most notably natural killer (NK) cell mediated cytotoxicity. Natural killer (NK) cells are considered the most effective antitumor cell within the bloodstream. In mice, metastasis increases when either NK cells are depleted [82, 83] or have decreased lytic ability due to a genetic defect[84]. Many studies have demonstrated that platelets are capable of protecting cancer cells from NK cells mediated cytolysis *in vivo* and *in vitro* [79, 80, 85-87]. Nieswandt *et al* showed that platelets are only required for successful metastasis if the tumour cell line studied are sensitive to NK cell mediated cytolysis [79]. Further, Palumbo *et al* showed the anti-metastatic effect of fibrinogen$^{-/-}$ and Gαq$^{-/-}$ in mice was eliminated by the immunologic or genetic depletion of NK cells[59]. It has been shown how platelets inhibit NK cell lysis of tumour cells. NK cell activity can be triggered by cells lacking expression of MHC (Major histocompatibility complex) class I molecules, a process termed 'missing self' recognition [88]. Placke *et al* have demonstrated that platelet adhesion to tumour cells impedes NK cell mediated cell lysis via the 'pseudoexpression' of immunoregulatory MHC class I molecules on the surface of tumour cells following platelet adhesion [86]. Kopp *et al* have also shown that platelet derived TGFβ (transforming growth factor β) released upon platelet activation can inhibit NK cell activity by downregulating the activation of the NK cell receptor NKG2D[89]. TGFβ is a growth factor stored at very high concentrations in platelet granules. The importance of TGFβ in platelet-cancer cell interactions has been confirmed by a recent study by Labelle *et al* showing that the genetic depletion of platelet derived TGFβ1 was protective against metastasis [90].

1.4.4 Platelets prevent haemorrhage at sites of tumour associated vasculature

More recently, it has come to light that platelets are important in maintaining the integrity of tumour blood vessels. Tumour vasculature is phenotypically abnormal, characterised by thin vessels walls, excessive branching, and turbulent flow patterns. These factors coupled with increased leukocyte infiltration and high levels of VEGF leading to increased vascular permeability, mean tumour blood vessels are exposed to constant damage[91-94]. Ho-Tin-Noe *et al* pharmacologically induced thrombocytopenia in tumour bearing mice, using a platelet depleting antibody[95]. They found that the onset of thrombocytopenia caused rapid (~ 30 minutes) severe bleeding specific to the site of the tumour, indicating platelets regulate tumour vessel stability. Thrombocytopenia induced bleeding was localised to sites of significant immune cell infiltration, and reduced in mice genetically deficient in leukocyte integrins. Bleeding was independent of tumour type, age, or location. It was seen in subcutaneous melanoma and breast cancer models, as well as melanoma pulmonary metastasis models.

Ho-Tin-Noe *et al* also studied the mechanism underlying the protective role of platelets[95]. Microscopy studies of tumour microvessels showed an absence of adherent platelets, suggesting the protective role of platelets was not dependent on platelet adhesion or aggregation. In line with this observation, $VF^{-/-}$ mice, GPIbα inhibitor treated mice, P-$selectin^{-/-}$ mice, and mice with defective αIIbβ3 activation (CalDAG-$GEFI^{-/-}$) showed no intratumour bleeding. Transfusion of GPIbα mutant or normal resting platelets corrected the bleeding phenotype seen in thrombocytopenic mice. However, transfusion of thrombin degranulated platelets failed to correct the bleeding phenotype, indicating the protective role of platelets on tumour vessel stability was dependent on the content of platelet granules and other factors (shed platelet receptors, procoagulant platelet microparticles) being released during platelet activation. However, administration of high doses of platelet releasate did not correct the tumour associated bleeding phenotype observed in thrombocytopenic tumour bearing mice. The mechanism of platelet

activation in tumour vessels, in the absence of platelet adhesion, and the identity of the factors responsible for the protective effect on vessel stability still remain to be determined[92]. In a mouse model, Demers *et al* have shown that thrombocytopenia improves the delivery of chemotherapeutic agents (paclitaxel) to tumours, causing the increased accumulation of the drug within the tumour and decreased proliferation of both slow and fast growing tumour[96]. This suggests that targeting platelet function may be useful in proving the efficacy of anticancer therapies[97].

1.4.5 Platelets as a target for anticancer therapies

Given the importance of platelets in the pathogenesis of cancer, at least in experimental models, there is no surprise that there is interest in the use of antiplatelet agents as an anti-cancer therapeutic strategy. Aspirin use has been associated with a decreased incidence of cancer and cancer related deaths, while the P2Y12 receptor antagonist prasugrel has been linked with an increased incidence of cancer.

1.4.5a Aspirin and cancer

Aspirin (Acetylsalicylic acid) is most widely administered antiplatelet agent. It elicits its anti-platelet effect by inhibiting prostaglandin biosynthesis, including the synthesis of the platelet agonist TxA2. Aspirin inhibits prostaglandin biosynthesis through the irreversible inactivation of Prostaglandin H (PGH) synthase 1 and synthase 2, more commonly referred to as COX-1 and COX-2. These enzymes catalyse the first step of prostaglandin biosynthesis, the conversion of free arachidonic acid into Prostaglandin H_2 (PGH_2) [98]. Aspirin induced acetylation of serine 529 in human COX-1 and serine 516 in human COX-2, blocks arachidonic acid from entering the COX catalytic site[99]. The PGH_2 generated by the cyclooxygenation of arachidonic acid serves as a substrate for downstream isomerases, leading to the production of at least five prostaglandins, including TxA_2. TxA_2 is synthesised by platelets in response to activation, and induces irreversible platelet aggregation via its G-protein coupled receptor, the TxA2 receptor [99-101].

Aspirin has a proven benefit in the treatment of cardiovascular disease. Over 100 trials comprising thousands of patients have demonstrated its efficacy in preventing major adverse cardiovascular events. Overall, in high risk patients groups, aspirin reduces vascular death by approximately 15 % and non fatal vascular events by approximately 30% [102]. There is also evidence to suggest that regular aspirin use reduces the incidence of certain types of cancer. The protective benefit of aspirin is well established for colon cancer. Randomised controlled trials of daily aspirin use

have shown a reduction in the incidence of colorectal cancer, recurrence of colorectal adenocarcinomas, and mortality form colorectal cancer [103-105]. Recently, meta-analyses of trials of aspirin for the prevention of vascular events have shown that regular aspirin use reduces the overall incidence of cancer, metastasis, and cancer related deaths. A 30% reduction in the incidence of cancer and a 40% decrease in mortality were observed at follow up. The beneficial effect of aspirin did not increase at concentrations greater that 75 mg/day but did increase with the duration of treatment, with the beneficial effect most evident after 3-5 years. The beneficial effect was not seen for all cancer types, but was particularly evident for adenocarcinomas [106-108]. However, this effect is not seen in all studies. The Women's Health Study, a 10 year long trial of 100 mg aspirin per day, showed no reduction in the incidence of cancer or mortality [109].

It has been suggested that aspirin elicits its inhibitory effect on cancer through COX-2 inhibition. However, this hypothesis is inconsistent with the results of various randomised trials and meta-analyses [110]. Extremely high doses of aspirin (up to 650 mg) 3 to 4 times daily are required for sustained COX-2 inhibition [111]. In contrast, the chemopreventive effect of aspirin on colon cancer occurs at low doses (75 mg/day) and does not increase with higher doses [104, 107, 108]. An alternative hypothesis is that aspirin elicits its anticancer effect through the permanent inactivation of platelet COX-1[110]. Several features of the protective effect of on cancer support this theory. Platelet COX-1 is sensitive to low doses of aspirin; 30mg/day completely inhibits prostaglandin synthesis in platelets [111-113]. Doses of aspirin at 24 hour intervals appear to be effective against cancer. Daily doses of aspirin are insufficient to inhibit COX-1 and COX-2 in nucleated cells. Aspirin has an extremely short half life and nucleated cells are able to generate COX enzymes within a few hours. In contrast, anuclear platelets cannot regenerate COX and the inhibitory effect of aspirin lasts the lifetime of the platelet[110]. Furthermore, the Thrombosis prevention trial (TPT) showed a reduction in the mortality due to gastrointestinal adenocarcinoma in patients prescribed a controlled release formulation of aspirin designed to inhibit platelet function but have negligible systemic effects[105, 114].

1.4.6b P2Y12 receptor antagonists and cancer

The other most widely used antiplatelet agents are the ADP-P2Y12 receptor antagonists. ADP is a relatively weak but important platelet agonist, stored in and secreted from platelet dense granules upon activation. It serves to amplify platelet aggregation in response to other agonist stimuli[115-117]. ADP acts through the G protein coupled receptors P2Y1 and P2Y12. Due to its limited tissue distribution compared to P2Y1, the P2Y12 receptor is an excellent target for antiplatelet therapy. P2Y12 antagonists are generally used in combination with aspirin producing a synergistic inhibitory effect on platelet activation and aggregation. Clinically, dual antiplatelet therapy provides a modest clinical benefit over aspirin in the prevention of major cardiovascular events[102, 118].

Recently, the P2Y12 inhibitor prasugrel has been associated with an increased incidence of cancer. This is based on the results of the Triton-TIMI 38 trial (Therapeutic Outcomes by Optimising Platelet Inhibition by Prasugrel-Thrombolysis in Myocardial Infarction 38). In this trial, prasugrel (60 mg loading dose, 10 mg daily) was compared with another P2Y12 antagonist clopidogrel (300 mg loading dose, 75 mg daily) in 13,608 patients with acute coronary syndromes undergoing percutaneous coronary intervention[119, 120]. Both clopidogrel and prasugrel are thienopyridines. Thienopyridines are pro-drugs (inactive *in vitro*), metabolised to short lived active metabolites by hepatic cytochrome P450. The active metabolites selectively and irreversibly bind to P2Y12 and subsequently inhibit ADP induced platelet aggregation[121, 122]. The metabolism of clopidogrel is highly dependent on the hepatic cytochrome P450 enzyme CYP2C19. A reduced function allele of CYP2C19 is extremely common. It is associated with the decreased metabolism of clopidogrel, reduced bioavailability of the active metabolite, and therefore reduced platelet inhibition [123]. The metabolism of prasugrel is less dependent on CYP2C19 and so is less affected by the reduced function allele. As a result, the metabolism of prasugrel is more effective, leading to greater bioavailability of the active metabolite, and therefore more consistent inhibition of platelet function[124, 125].

In the TRITON-TIMI 38 trial, the primary composite end point was death from cardiovascular causes, non fatal myocardial infarction, or nonfatal stroke. Prasugrel was associated with a significant decrease in the primary end point (12.1 % vs. 9.9%, $p < 0.0001$,), but was also associated with a significant increase in major bleeding (1.8 % vs. 2.4 %, $p = 0.03$), including life threatening bleeding (0.9% vs. 1.4 %, $p = 0.01$). An analysis of the TRITON-TIMI 38 study by the FDA showed an increase in various types of solid tumours associated with prasugrel use. However, *in vitro* studies with human cancer cell lines or *in vivo* studies of human cancer cells implanted in mice showed prasugrel did not promote tumour growth or proliferation [126].

This led some commentators to speculate that prasugrel promotes cancer through its antiplatelet effect. Floyd and Serebruany suggested platelets provide a natural barrier against dissemination by trapping tumour cells in the microcirculation and inhibiting angiogenesis through the release of platelet factor 4[127]. They suggest that prasugrel is a more potent inhibitor of platelet function than clopidogrel and therefore more effectively inhibits the natural platelet defence against cancer growth and dissemination. However, this hypothesis is inconsistent with the published literature. Prasugrel is not a more potent inhibitor of platelet function than clopidogrel, rather it pharmacokinetics are better. It is metabolised more effectively leading to greater bioavailability of the active metabolite. P2Y12 function is completely inhibited in all prasugrel patients but only in approximately 75% of clopidogrel treated platelets[128]. There is no increase in the incidence of cancer in clopidogrel treated patients who are fully inhibited. In the CAPRIE (Clopidogrel vs. Aspirin in Patients at Risk of Ischemic Events) trial, the incidence of solid tumours was similar in clopidogrel, aspirin, and placebo treated patients[129]. In the CHARISMA (Clopidogrel for High Atherothrombotic Risk and Ischemic Stabilisation, Management, and Avoidance) trial, the incidence of solid tumours was actually lower in the clopidogrel group compared to the aspirin groups[130]. Furthermore, in the PLATO (Platelet Inhibition and Patient Outcomes) trial, the direct P2Y12 antagonist ticagrelor was associated with a significant decrease in the rate of cardiovascular death, and cardiovascular events, as well as all cause mortality, when

compared to clopidogrel [131]. These results suggest that increased inhibition of platelet function through P2Y12 receptor blockade is not associated with an increased incidence of cancer.

1.5 Cancer associated thrombosis

The activation of the haemostatic system in cancer patients also manifests itself in the increased incidence of thrombosis in this cohort. Thrombosis is a frequent complication of cancer and is the second leading cause of death in cancer patients after metastasis. This link between cancer and thrombosis has been known for nearly two centuries now. Early observations described the high rate of thrombosis in patients with cancer. The French physician Armond Trousseau is commonly thought to be the first person to describe this phenomenon. In 1865, he reported a high incidence of venous thrombosis in patients with gastric cancer. However, 30 years prior, Bouillard had already described deep vein thrombosis (DVT) in 3 patients with cancer[132-136].

Clinically detectable thrombosis is seen in 15 % of cancer patients [136-138]. Up to 50% of patients who have died of non thrombotic complications also show subclinical evidence of thrombosis upon autopsy [133, 139]. Cancer patients are at increased risk of recurrent thrombosis compared to non-cancer patients, even when on oral anticoagulant therapy[140, 141]. Patients with idiopathic thrombosis (thrombosis of unknown cause) are considered at high risk for developing or being subsequently diagnosed with cancer [135, 142]. DVT of the leg is the most common clinical manifestation of thrombosis in cancer patients. However, DVT of the arm, pulmonary embolism, cerebral sinus thrombosis, disseminated intravascular coagulation, non bacterial thrombotic endocarditis, and arterial thrombosis have all been documented in cancer patients to a lesser extent [143-147].

In patients with cancer, tumour site, tumour stage, therapy (chemotherapy, radiotherapy, hormonal therapy), and surgery are the major determinants of thrombosis [148]. Virchow's triad defines the 3 broad categories of factors that underlie thrombosis. These factors are stasis *(reduced or disturbed blood flow)*, vascular damage *(damage to the endothelial lining of blood vessels)*, and the hypercoagulability of blood *(increased platelet and coagulation activity)*. In cancer patients, all 3 factors are altered due to the underlying disease or treatment

strategies and hence, contribute to the increased incidence of thrombosis in patients with cancer (Table 1.1).

Stasis: Blood stasis caused by reduced patient mobility and vascular compression due to bulky tumours can contribute to thrombosis. For example, direct tumour ingrowth into the renal vein or vena cava is observed in 5-10 % of renal cancer patients [149]. ***Vascular damage:*** Intravasating and extravasating cancer cells, release of inflammatory cytokines (e.g. Il-6) by tumours, chemotherapeutic agents, and central venous catheters can all contribute to endothelial cell activation and vascular damage [150]. For example, the chemotherapeutic agent cisplatin has been associated with venous thromboembolism, stroke, recurrent peripheral arterial thrombosis, and aortic thrombosis [150-152]. Cisplatin therapy is associated with elevated levels of von Willebrand factor (released from the Weibel Palade bodies of endothelial cell) suggestive of endothelial cell activation[153, 154].

Blood hypercoagulability: The release of procoagulant factors and platelet agonists by tumours, direct interactions between cancer cells and blood cells, and the effect of chemotherapeutic agents on coagulation proteins and platelets can contribute to hypercoagulability[136, 155, 156]. For example, cancer procoagulant is a cysteine protease that directly activates factor X, eventually leading to thrombin generation and fibrin formation. Increased cancer procoagulant expression levels have been reported in patients with colon, breast, lung and renal cancer, as well as leukaemia and melanoma patients [136, 157].

Table 1.1 Virchow's Triad in patients with cancer. Venous stasis (reduced or perturbed blood flow), vascular damage, and blood hypercoagulability are the three factors that contribute to thrombosis. In cancer patients, all 3 factors are altered due to underlying disease or treatment strategies and hence, contribute to the increased incidence of thrombosis in patients with cancer. Adapted from (Petralia et al[158])

Venous Stasis	Endothelial Injury/ Alteration in Blood Vessels	Hypercoagulable State
• Increased blood viscosity • Mechanical blockage (tumor extrinsic compression or invasion) • Patient immobility (due to cancer complications or treatment)	• Mechanical endothelial trauma (due to cancer invasion or treatment) • Endothelial dysfunction/loss of antithrombotic properties • Angiogenic stimuli	• Increase in procoagulant activities • Decrease in anticoagulant activities • Increase in overall platelet activity • Decrease in fibrinolytic activity

1.6 Thesis outline and hypotheses

This thesis is focussed on the role of platelets in the pathology of cancer. It explores different platelet-cancer interactions that could potentially influence the pathology of cancer *in vivo*, ranging from platelet induced signalling in ovarian cancer cells to platelet reactivity in patients with malignant disease.

Chapter 3: Platelet adhesion and degranulation induce pro-angiogenic, anti-apoptotic signalling in ovarian cancer cells

Platelet – tumour cell interactions have been shown to be critical for metastasis. Multiple tumour cell lines from different origins are capable of supporting platelet adhesion and inducing platelet activation. These events (platelet adhesion and degranulation) have been shown to be beneficial for tumour cells *in vitro* and *in vivo*. For example, platelet adhesion to tumour cells (or 'platelet cloaking') and tumour cell induced platelet degranulation, specifically the release of TGFβ, inhibit NK cell activity [59, 79, 85, 86, 89]. However, whether platelet adhesion and degranulation induce direct tumour cell signalling events that could influence disease progression is not known.

In the case of endothelial cells and the progression of atherosclerosis, it is well known that platelet induced signalling influences disease progression. Atherosclerosis is a complex chronic inflammatory condition affecting the arterial circulation. It results in the development of atherosclerotic plaques, asymmetrical thickenings of the intima of muscular and elastic arteries, consisting of extracellular matrix proteins, lipids, monocyte derived macrophages, T-lymphocytes, and smooth muscle cells [159, 160]. It has been demonstrated that platelets adhere to lesion prone sites in $ApoE^{-/-}$ dyslipademic mice even in the absence of visible atherosclerotic lesions. Furthermore, the infusion of activated platelets into In $ApoE^{-/-}$ mice causes an increase in atherosclerotic lesion size [161]. Activated platelets have been shown to modulate the chemotactic and adhesive properties of endothelial cells *in vitro*, by inducing the release of MCP-1 (monocyte

chemoattractant protein 1), and the increased surface expression of ICAM-1 (intercellular cell adhesion molecule 1) in an NF-κB (nuclear factor κB) dependent mechanism. The accumulation of monocytes at sites of endothelial cell activation or inflammation is one of the driving events in the development of atherosclerosis. MCP-1 is a potent chemotactic of monocytes but not neutrophils, while ICAM-1 can mediate monocyte adhesion to the endothelium [162]. Hence, platelets can induce signalling in endothelial cells that can influence disease progression.

It was hypothesised that platelet interactions with tumour cells could also induce signalling events in tumour cells that could influence disease progression. Using ovarian cancer cells as a model, the effect of platelet adhesion and degranulation on a range ovarian cancer cell lines was assessed. Ovarian cancer cells were shown to support platelet adhesion and induce platelet degranulation. Subsequently, ovarian cancer cells were incubated with washed platelets (*representing platelet adhesion)* or platelet releasate *(representing platelet degranulation)* and gene expression profiling was performed. Platelet adhesion and degranulation events were shown to induce anti-apoptotic and pro-angiogenic signalling in ovarian cancer cells that could potentially modulate their metastatic potential *in vivo.* Hence, platelets can induce signalling events in tumour cells that could potentially promote disease progression.

Chapter 4: Living in shear – platelet protection in the circulation

During bloodborne metastasis, circulating tumour cells are exposed to the negative pressures of the circulation, immune surveillance and shear forces. It is well known that platelets facilitate metastasis, in part, by protecting tumour cells from immune surveillance. Platelet cloaking of tumour cells (or platelet adhesion to tumour cells) has been convincingly shown to prevent NK cell mediated cytotoxicity of tumour cells *in vitro* and *in vivo*[59, 79, 85, 86, 89]. It is also frequently suggested that platelets protect from tumour cells from shear stress, a biomechanical force generated by

blood flow[74, 90, 163]. However, currently, there is no evidence to support or oppose this hypothesis.

It was hypothesised that through the phenomenon of platelet cloaking (platelet tumour cell adhesion), platelets would act as a physical shield around tumour cells, and hence protect them against shear forces. To assess this, A2780 ovarian cancer cells were exposed to physiologically relevant venous and arterial shear rates in the presence or absence of platelets and LDH release was used to assess mechanical damage to the A2780 cell membrane. Platelets were associated with a significant decrease in LDH release by A2780 cells under shear conditions, suggesting that platelet cloaking can protect tumour cells from shear induced damage under physiologically relevant conditions.

Chapter 5: Platelet hyperreactivity in metastatic cancer patients

Patients with cancer are at increased risk of thrombosis, a factor that contributes to mortality and morbidity in this cohort. In patients with cancer, tumour site, tumour stage, therapy (chemotherapy, radiotherapy, hormonal therapy), and surgery are the major determinants of thrombosis. However, modulation of the haemostatic system during cancer progression is believed to make a significant contribution to thrombosis in this cohort [148]. It is well established that patients with cancer show increased coagulation activation, as evidenced by increased levels of fibrinogen breakdown products and coagulation proteins [164-167].

In contrast, there is a paucity of studies assessing whether platelet function is altered in cancer patients, despite their importance in thrombosis. In response to physiological agonists, platelets become activated (e.g. release of granule content, integrin αIIbβ3 becomes activated), then aggregate together. These events can be measured *ex vivo* using a number of platelet function assays. Based on platelet function testing, the concept of platelet hyperreactivity has been recognised and can be defined as an exaggerated platelet response (e.g.) increased platelet activation or

aggregation in response to low doses of platelet agonists [168]. Rather than being a laboratory artefact, measures of increased platelet reactivity, for example, increased αIIbβ3 activation[169], increased spontaneous platelet aggregation [170], and increased ADP and epinephrine induced platelet aggregation [171, 172], have been associated with an increased incidence of major adverse cardiovascular events.

In line with abnormalities in secondary haemostasis, it was hypothesised that patients with cancer would display increased platelet reactivity, a factor that could contribute to the increased incidence of thrombosis in this cohort. To assess this hypothesis, blood samples were collected from 13 patients with advanced metastatic cancer and 10 healthy controls and platelet function testing was performed. Patients with metastatic cancer were chosen since abnormalities in coagulation are known to increase with disease progression, with 90% of patients with metastatic cancer showing abnormalities in coagulation [167]. Hence, patients with advanced disease (of any origin) would likely have more overt abnormalities in platelet reactivity compared to patients with localised disease. In this study, it is clearly demonstrated that patients with metastatic cancer display significantly increased platelet reactivity, as evidenced by increased agonist induced platelet aggregation, increased spontaneous platelet aggregation, and increased agonist induced platelet activation (P-selectin expression).

Chapter 2: MATERIALS AND METHODS

2.1 Reagents

EDTA (ethylenediamine tetra acetic acid), bovine serum albumin (BSA), sodium citrate, D-Glucose, Triton X, Streptomycin, Penicillin, MRS2159, MRS2179, Trypsin-EDTA, Cisplatin, Medium 199, MCDB-105 media, Prostaglandin E1, HEPES, PAR-1 activating peptide, PAR-4 activating peptide, Sodium bicarbonate, potassium phosphate, potassium chloride, apyrase, Trisma base, magnesium chloride (hexahydrate), calcium chloride, citric acid, and TOX-7 cytotoxicity assay were purchased from Sigma -Aldrich (Tallaght, Ireland). Aggrectin (Ristocetin sulphate), Collagen (soluble calf skin), Adenosine-5'-Diphosphate, Epinephrine, and Arachidonic Acid were obtained from BioData (Horsham, PA, USA). Alexa Fluor-488-labelled Phalloidin, Calcein AM, Annexin V, and fibrinogen were obtained from Invitrogen (Carlsbad, CA, USA). Phycoerythrin (PE)-labelled anti human P-selectin (mouse IgG), PE-labelled mouse IgG isotype control, PE-labelled anti human CD42a (mouse IgG), APC-labelled anti human CD42b (mouse IgG), APC labelled mouse IgG control , PAC-1, and FITC-labelled mouse IgG isotype control antibodies were purchased from BD Pharmigen (San Diego, CA, USA). DMEM (Dulbecco's modified eagles medium), PBS (phosphate buffered saline), RPMI 1640 media (Roswell Park Memorial Institute), McCoy's 5a media, and FBS (foetal bovine serum) were purchased from Biosera Ltd (Sussex, UK). Coverslips and 96 well flat bottomed plates were purchased from Fisher Scientific (Dublin, Ireland). Poly-l-lysine coated slides were purchased from VWR international. Chrono-lume reagent was purchased from Chrono-log (UK). Recombinant Hirudin (Refludan) was purchased from Pharmion (Celegene Corporation, NJ, USA).

2.2 Ethics Statement

Blood collection was approved by the Royal College of Surgeons in Ireland and Beaumont Hospital ethics committee and written informed consent was obtained from all donors prior to phlebotomy.

2.3 Platelet Preperation

Blood was obtained from volunteers in RCSI or Beaumont hospital who had not taken medications known to affect platelet function for at least 10 days. Blood was collected by venipuncture through a 19-gauge butterfly needle without a tourniquet, to avoid platelet activation. For the preparation of citrated platelet-rich plasma (PRP), blood was collected into a syringe containing 3.2% trisodium citrate as anticoagulant (10% vol/vol), then centrifuged at 170g for 10 minutes at room temperature. Citrated platelet poor plasma (PPP) was prepared by centrifuging the remaining whole blood at 1500g for 10 minutes at room temperature. For the preperation of hirudinised PRP, whole blood was taken into a syringe containing 300 U/ml of recombinant hirudin then centrifuged for 10 minutes at room temperature. For the preparation of washed platelets, blood was collected into Acid-Citrate-Dextrose (ACD: 38mM citric acid, 75mM sodium citrate, 124mM D-glucose) as anticoagulant (15% vol/vol). Blood was centrifuged at 170g for 10 minutes at room temperature. PRP was acidified to pH 6.5 with ACD, and PGE_1 (1 µM) was added to avoid platelet activation during centrifugation. Platelets were pelleted by centrifugation at 720g for 10 minutes. The supernatant was removed and the platelet pellet was resuspended in JNL buffer (130 mM NaCl, 10 mM sodium citrate, 9 mM NaHCO3, 6 mM D-glucose, and 0.9 mM MgCl2, 0.81 mM KH2PO4, and 10 mM Tris, pH 7.4) to a concentration of 2.5 x 10^8/mL and supplemented with 1.8 mM $CaCl_2$.

2.4 Cell Culture

The non tumorigenic ovarian epithelial cell line HIO-80 (a gift from the Fox Chase Cancer Center, Philadelphia, PA) was established by transfection of normal ovarian epithelial cells with a plasmid encoding for the SV40 large T gene. The HIO-80 cells were grown in a 1:1 mixture of medium 199 and MCDB-105 supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 units/ml penicillin, and 100 ug/ml streptomycin. The metastatic ovarian cancer cell line 59M (European Collection of cell cultures (ECACC), Salisbury, UK) was established from the ascites of a patient with an ovarian carcinoma. 59M cells were cultured in DMEM supplemented with

2mM glutamine and 10 % FBS. The metastatic ovarian cancer cell line SK-OV-3 (American Type Culture Collection (ATCC), Manassas, VA, USA) was isolated from the ascites of a 64 year old patient with a tumorigenic human ovarian epithelial adenocarcinoma. SKOV-3 cells were grown in McCoy's 5A media, supplemented with 10% fetal bovine serum, 100 units/ml penicillin and 100 ug/ml streptomycin. The ovarian cancer cell line A2780 (ECACC, Salisbury, UK) was established form the primary tumor tissue of a female patient with an ovarian carcinoma. The cisplatin-resistant cell line A2780cis was developed by chronic treatment of the parent cisplatin-sensitive A2780 cell line with increasing concentrations of cisplatin. Both cell lines were grown in RPMI 1640 media supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 units/ml penicillin and 100 ug/ml streptomycin. All cells were grown in a 37° C humidified atmosphere containing 5% CO2.

Table 2.1 Cell line information. 5 ovarian cancer cell lines were used in this study. They ranged from an immortalised ('normal') ovarian epithelial cell line (HIO-80) to metastaic cancer cells lines (SKOV-3 and 59M).

Cell line	Origin	Cell type	Post isolation treatments
HIO-80	Normal Ovarian tissue	Epithelial	Immortalised by transfection with a plasmid encoding for the SV40 large T gene
A2780	1° ovarian tumour tissue	Epithelial	-
A2780cis	1° ovarian tumour tissue	Epithelial	Grown in cisplatin t o select for resistant cells
SKOV-3	Ascites of patient with metastatic ovarian cancer	Epithelial (adenocarcinoma)	-
59M	Ascites of patient with metastatic ovarian cancer	Epithelial	-

2.5 Preperation of cancer cells suspensions

When 70-80% confluent, cells were detached with 5 ml of 7 mM EDTA in Dulbecco's PBS (5 minutes at 37 °C). 5 ml of JNL buffer was added and the cells were washed twice by centrifugation (180 g for 5 minutes). After the second wash, the cells were resuspended in JNL supplemented with 1.8mM $CaCl_2$, counted using a haemocytometer (with trypan blue exclusion of dead cells) and eventually diluted to the desired concentration.

2.6 Quantifying platelet adhesion to cancer cells under static conditions

Platelet adhesion assay to cancer cells was measured using as previously described method. Cancer cells (5 x 10^4/ml) were seeded in 96 well plates (Nunc, Thermo Fisher Scientific, Braunschweig, Germany) and grown until confluent. Washed platelets (1.5 x10^8/ml) preloaded with Calcein AM (2 µg/ml, 45 minutes) were allowed to adhere to the cells for 45 minutes at 37°C. The total fluorescence (485/535 nm) per well was measured using a Wallac 1420 Victor2™ multilabel counter (Perkin Elmer, Waltham, MA., USA) plate reader. The plate was washed three times, 100 µl of JNL was added to each well of the plate, and the remaining fluorescence was read. % platelet adhesion was calculated as the (remaining fluorescence – blank)/ (total fluorescence – blank) x 100.

2.7 Imaging platelet adhesion to cancer cells under static conditions

Platelet adhesion to cancer cells was imaged by flourescence microscopy. Cells (1x10^5/ml) were incubated on a BSA coated poly-L-lysine slide for 45 minutes at 37 °C. Following adhesion, washed platelets (50 x 10^3/µl) were added to the slide and incubated for 45 minutes at 37 °C. Samples were fixed with 3.7% paraformaldehyde for 15 minutes at room temperature and permeabilised with 0.1% Triton X-100. Platelets and cancer cells were stained with Alexa Fluor-488 Phalloidin (41.25 nM) for 15 minutes at room temperature. Platelets were specifically stained with

phycoerythrin labelled anti-human CD42a antibody (1.25 µg/ml) for 2 hours at 37 °C. The slides were mounted and imaged by fluorescence microscopy. The slides were washed with JNL buffer (x 3) between all steps.

2.8 Tumour cell induced platelet activation (P-selectin expression and PAC-1 binding)

The ability of cancer cells to induce platelet activation (P-selectin expression) was assessed using a flow cytometry based assay modified from Nylander *et al* [173]. In a total reaction volume of 100 µl, 10 µl of PRP was incubated with cancer cells (0 – 1.5 x 10^6/ml) in the presence of a PE-labelled anti human P-selectin and a FITC labelled PAC-1 (binds activated integrin αIIbβ3) antibody (1.25 µg/ml) or an appropriate isotype control (1.25 µg/ml). All incubations were performed at room temperature for 15 minutes. The reaction was then terminated with 1 ml of JNL buffer prior to analysis by flow cytometry. Samples were analysed using a BD FACS Calibur (Becton Dickinson, Palo Alto, CA, USA) within 1 hour. The instrument was set to measure size (forward scatter, FSC), granularity (side scatter, SSC) and cell flourescence. Using a log FSC vs. log SSC dot plot, a two dimensional analysis gate was drawn around the platelet population, and a flourescence histogram (log FL1 or FL2 vs. count) was obtained for 10000 platelet events for each sample. Data was analysed using CellQuest Pro software and expressed as percentage of platelets that were P-selectin positive relative to the isotype control.

2.9 Isolation of platelet releasate

To induce full platelet degranulation, washed platelets (2.5 x 10^8/ml) were stimulated with PAR-1 activating peptide (20 µM) and Collagen (190 µg/ml) and stirred in a BioData PAP-4 light transmission aggregometer (Horsham, PA, USA) at 37 °C for 10 minutes. The platelet aggregate was centrifuged at 720g for 10 minutes. The supernatant was then aspirated, syringe filtered to remove microparticles (0.22 µm pores), and stored at -20 ° C until use.

2.10 MTT assay

To optimise the concentration of platelet releasate for tumour cell exposure, a series of MTT cell proliferation assays (Roche Diagnostics Ltd, United Kingdom) were performed according to the manufacturer's instructions to determine the maximum concentration that could be applied to cells without negatively impacting on cell growth or survival. Cells were seeded into 96-well cell culture plates at 2×10^4 cells/well and cultured for 24 hours to allow cell attachment. Once attached to the plates, growth media was aspirated and cells were briefly washed with 200 µl of pre warmed PBS. Cells were then exposed to serial dilutions of platelet releasate from 1:10 to 1:10,000 in full media, serum free media or JNL buffer to allow optimisation of concentration to be used in subsequent gene expression based studies.

2.11 Washed platelet/platelet releasate exposure for gene expression analysis

The optimal concentration of platelet releasate (determined by MTT) was applied to the cell lines and the levels of apoptosis were compared against cells grown in full media only using a Roche Apodirect TUNEL/Propidium iodide kit. As the 1:1000 dilution of platelet releasate in full growth media was observed to be the highest concentration of platelet releasate in full media that did not impact on cell growth/viability for all cell lines and was subsequently shown to result in no significant difference in apoptosis compared to cells grown in full media alone it was determined that it was suitable to proceed with this concentration. Given that the platelet releasate was prepared from identical washed platelet preparations, the 1:1000 dilution of platelet releasate directly informs the use of a 1:1000 dilution of washed platelets for comparative study. Optimal concentrations of platelet releasate and washed platelets (1:1000) were suspended in full culture media and applied to cultured cells in 75 cm^2 flasks in triplicate. Cells were exposed to releasate or washed platelets for 6 hours after which total RNA was extracted from cells. Transcriptome analysis was performed using Affymetrix Human Exon Arrays.

2.12 RNA extraction

Cells were washed briefly in PBS, trypsinised and centrifuged to remove supernatant. RNA was extracted using RNeasy mini kit (Qiagen Ltd., West Sussex, UK) according to the manufacturer's protocol. RNA quantity was assessed using a Nano-Drop ND-1000 Spectrophotometer (Wilmington, USA) and quality by an Agilent Bioanalyser 2100 and RNA 6000 Nano microfluidic chip assay (Santa Clara, USA). RNA was stored at –80°C.

2.13 Affymetrix array

100 ng of total RNA extracted from control cells and cells exposed to washed platelets/platelet releasate was labelled using the Ambion WT Expression kit (Ambion/Life Technologies, Austin, TX, USA) including the labelling controls from the Affymetrix Gene Chip Poly-A RNA Control Kit. As suggested by Affymetrix/Ambion, at each step of the sample preparation protocol, progress was monitored using both the Agilent 2100 Bioanalyzer and the Nanodrop spectrophotometer. Quality control (QC) required assessment after the first cycle RNA cleanup, after the second cycle single-strand cDNA cleanup and following ssDNA fragmentation. Prepared fragmented ssDNA was hybridised to the Affymetrix Human Exon 1.0 ST Array at 45°C for 16 hours following Affymetrix protocols for their GeneChip WT Terminal Labeling, GeneChip Hybridization Control and GeneChip Hybridization, Wash, and Stain kits (Affymetrix, Santa Clara, USA). Following hybridization, the chips were washed and stained using the Affymetrix GeneChip Fluidics Station with appropriate 64 format assay protocol. Following staining and washing steps the Affymetrix GeneChip Scanner 3000 and Affymetrix GeneChip Operating Software was used for the management and initial processing of the expression data. The data from 45 exon arrays was subject to array quality control performed using the Affymetrix Expression Console. All controls were within the parameters suggested by Affymetrix. Following successful quality control standards assessment, the chip data was then analysed in depth using Biotique Systems XRAY analysis software. Each cell line was examined under three different conditions; resting cells, cells exposed to platelet releasate, and cells exposed to washed platelets. Each cell line and condition

was assayed in triplicate. The software was used to compare the two exposure cohorts against the resting control and genes exhibiting a positive or negative fold change of greater than 1.5 and a significance of $p \leq 0.05$ examined.

2.14 Fluidigm validation

Gene expression was validated using Fluidigm's high throughput qPCR 48.48 dynamic array system. Genes displaying the most significant fold changes in addition to genes involved in biologically relevant pathways were selected for validation. TaqMan® real-time PCR expression assays were used in conjunction with Fluidigm's microfluidic Biomark system. Samples were analysed in triplicate and results were compared with Affymetrix expression data. RNA was reverse transcribed to single stranded cDNA using a High Capacity cDNA RT Kit (Applied Biosystems, CA, USA) in 100µl reactions. Reactions contained 10 µl of buffer (10×), 4 µl of deoxynucleotide triphosphate (25×), 10 µl of random primers (10×), 5 µl of multiscribe RT enzyme (50 U/µl), 21 µl of nuclease-free water and 50 µl of extracted total RNA (20 ng/µl). Prior to Fluidigm, high throughput qPCR, a pre-amplification step was performed following Fluidigm protocols; 1 ml of TaqMan assay for each of the genes of interest identified through XRAY analysis and endogenous controls were pooled and made to a final volume of 100 ml in 1× TE Buffer, pH 8.0. 1.25 ml of each sample was added to 2.5 ml Preamp Master Mix (AB) and 1.25 ml pooled assay mix to give a 5 ml reaction volume. This mixture was then subject to 14 amplification cycles of 95°C, 15 seconds and 60°C, 4 minutes before being diluted 1:5 with DNAse and RNase free H_20 to give a final volume of 25 ml of pre-amplified cDNA. Fluidigm data was analysed using Fluidigm Real-Time PCR Analysis software (ver3.02) to yield relative quantitation values calibrated to normal (HIO-80) cells. Fold changes returned from Affymetrix analysis were plotted against those calculated from Fluidigm data and the correlation between the values was calculated using Graph Pad Prism (ver 5.02).

2.15 Lactate dehydrogenase release: Sigma TOX7 toxicology assay

The TOX-7 cytotoxicity kit (Sigma) was used to assess lactate dehydrogenase release in cancer cells and platelets. LDH is present in the cytoplasm of all cells. Upon membrane damage, it leaks into the cell supernatant. This 96 well plate colorimetric assay is based on the reduction of NAD by LDH. The reduced NAD (NADH) is used in the stoichiometric conversion of a tetrazolium dye. This coloured dye is measured spectrophotometrically. The assay kit consists of 3 reagents, cofactor preparation, dye solution, and substrate. These reagents are mixed in a 1:1:1 ratio. The LDH assay mixture is added to the sample of interest in a volume equal to twice the volume of the sample of interest being tested. The reaction is incubated for 20-30 minutes in the dark at room temperature. The absorbance is then measured at 490nm.

2.16 Optimisation of cells concentrations for LDH release

Based on the TOX-7 kit guidelines, the optimal concentration for LDH release for each cell type must be determined. The optimal concentration of cells is the concentration that gives the biggest increase in LDH relative to resting cells. For all cell lines used, serial dilutions of the cells from 0 – 1 x 10^6/ml (A2780 and 59M) and 0 - 250 x 10^6/ml (platelets) were prepared. For each concentration tested, a corresponding sample was treated with the lysis buffer supplied with the TOX-7 kit. The resting or lysed cells were incubated for 10 minutes at room temperature. The samples were centrifuged at 250g for 5 minutes and the supernatant was aspirated. 50 µl of each supernatant sample were aliquoted into the wells of a 96 well, clear, flat bottomed plate. The LDH assay mixture was prepared and 100 µl was added to each well of the plate. The plate was incubated for 25 minutes in the dark at room temperature. Absorbance values at 490nm were measured using a Victor^2 V Multilabel Counter plate reader (PerkinElmer, Wellesley, MA, USA). For each cell concentration, the absorbance values for resting cell samples were subtracted from absorbance values for lysed samples. The concentration of cells that gave the largest difference in LDH levels comparing the resting and lysed samples was chosen for

future experiments. The optimal cell concentrations determined were 1 x 10^6/mL for A2780 cells, 0.125 x 10^6/ml for 59M cells, and 250 x 10^6/ml for platelets (Figure 4a).

2.17 Cone and Plate Viscometry

Platelets and cancer cells were exposed to various shear rates using a cone and plate viscometer (Haake RheoStress 600, Thermo Fisher Scientific., Braunschweig, Germany). This device consists of a flat stationary plate and a rotating cone with a very obtuse angle. The sample of interest is placed in the centre of the plate. The cone is brought towards and stops at a fixed distance from the plate. The apex cone touches the plate but both are separated by a small gap at the edge. The sample fills this gap. The geometry of the cone and plate (cone angle and gap width) allow a uniform fixed shear rate to be applied across the sample. In this study, a cone angle of 5° and a gap width of 0.026 mm were used. Washed suspensions of cancer cells (1 x106/ml) or platelets (250 x 10^6/ml) were prepared. 600 µl of cells were added to the viscometer plate and exposed to shear conditions representing venous ($200s^{-1}$) and arterial ($1500s^{-1}$) blood flow for 1, 5, and 10 minutes at 37°C. Following this, 200µl of the sample was collected, centrifuged at 250g for 5 minutes, and the supernatant was aspirated. 50 µl of the supernatant was added to wells of a 96 well plate in duplicate and LDH release was quantified as described previously. To allow for slight increases in LDH release in resting cells with time, corresponding resting samples were prepared for each viscometry run. Resting and lysed samples were used to calibrate % LDH release under shear conditions. Lysed samples represented 100% LDH release. Resting samples represented 0% release. % release is calculated as (sheared cells – resting cells)/ (lysed cells-resting cells) x 100. To test the hypothesis that platelets can protect cancer cells from shear induced damage, washed suspensions of cancer cells were incubated with platelets in a ratio of 1:50 or 1:200 (cancer cell: platelet). 600 µl of the mixture was exposed to $200s^{-1}$ and $1500s^{-1}$ for 10 minutes at 37°C. Following this, 200 µl of the sample was collected, centrifuged at 250g for 5 minutes, and the supernatant was aspirated. 50 µl of the supernatant was added to wells of a 96 well plate in duplicate. LDH release was quantified and % release was calculated as above.

2.18 Quantifying platelet adhesion to cancer cells in suspension

Platelet adhesion to cancer cells was measured by flow cytometry. The assay is based on the detection of a platelet specific marker (GPIbα) on the surface of cancer cells following platelet adhesion. Washed suspensions of cancer cells (1 x 10^6/ml) were mixed with platelets (50 or 20 0 x 10^6/ml), then incubated for 1 minute (37 ° C) under sheared ($200s^{-1}$ and $1500s^{-1}$) or non sheared ($0s^{-1}$) conditions. Samples were washed to remove unbound platelets (180 g/10 minutes), fixed with 3.7 % Paraformaldehyde (15 min, room temperature), blocked in a 1% BSA solution to stop non specific antibody binding (1 hour, room temperature), and then stained with either an APC labelled mouse anti human CD42b or isotype control (APC mouse IgG1κ, 1 hour, room temperature). APC labelled antibodies were used as cancer cells are highly auto fluorescent at most wavelengths, but only minimally at wavelengths used to excite APC labels. Samples were analysed by flow cytometry using a BD FACS Canto (Becton Dickinson, Palo Alto, CA, USA). This flow cytometer is equipped with a laser to excite APC labels, whereas the previously used BD FACs Calibur is not. The instrument was set to measure size (forward scatter, FSC), granularity (side scatter, SSC) and cell fluorescence. Using a log FSC vs. log SSC dot plot, a two dimensional analysis gate was drawn around the tumour cell population. The fluorescence profile of 10000 events in this gate was obtained. Data was analysed using BD FACS DIVA™ software. % platelet tumour cell adhesion was calculated as the % of cells within the tumour cell gate positive for the platelet specific marker CD42b relative to the isotype control.

2.19 Imaging platelet adhesion to cancer cells in suspension

Platelet adhesion to cancer cells in suspension was imaged by fluorescence microscopy. Washed suspensions of platelets (200 x 10^6/ml) and cancer cells (1 x 10^6/ml) were prepared and mixed in ratio of 1:200 (cancer cell: platelets). The sample was incubated for 10 minutes (37°C) under sheared ($200s^{-1}$) or non sheared ($0s^{-1}$) conditions. Samples were fixed with 3.7 % paraformaldehyde (15 min, room temperature) and allowed to adhere to a poly-l-lysine coated slide for 15 minutes at

37° C. Samples were fixed to the slide with 3.7% paraformaldehyde for 15 minutes at room temperature and permeabilised with 0.1% Triton X-100 (3 minutes, room temperature). Platelets and cancer cells were stained with Alexa Fluor-488 Phalloidin (41.25 nM) for 15 minutes at room temperature. Platelets were specifically stained with phycoerythrin labelled anti-human CD42a antibody (1.25 µg/ml) for 2 hours at 37 °C. The slides were mounted and imaged by fluorescence microscopy. The slides were washed with JNL buffer (x 3) between all steps.

2.20 Platelet Aggregometry

Platelet aggregation responses were measured using a 96 well plate modification of classical light transmission aggregometry. Variable concentrations of agonists were prepared in a flat bottomed 96 well plate through the serial dilution of stock agonist concentrations in JNL buffer (130 mM NaCl, 10 mM sodium citrate, 9 mM $NaHCO_3$, 6 mM D-glucose, and 0.9 mM $MgCl_2$, 0.81 mM KH_2PO_4, and 10 mM Tris, pH 7.4). Platelet rich plasma was prepared and aliquots of 180µl of PRP were dispensed into wells of the plate containing 20µl of agonist, to give a final volume in each well of 200µl. The resulting concentrations of agonists on the plate were as follows : Arachidonic Acid (500, 375, 187.5, 93.8, 46.8, 23.5, 11.8,and 5.8 µg/mL), Collagen (190, 142.5, 71.25, 35.6, 17.8, 8.9, 4.4, and 2.2 µg/ml), ADP (20, 10, 5, 2.5, 1.25, 0.6, 0.3, 0.15 µM), Epinephrine (20, 5, 1.25, 0.3, 0.07, 0.01, 0.004, and 0.001 µM), and PAR-1 activating peptide (20, 10, 5, 2.5, 1.25, 0.6, 0.3, and 0.15 µM. Positive and negative controls were included in the form of 180µl of PRP/ PPP added to 20µl of JNL buffer. During the assay run, the plate was incubated at 37° C and shaken vigorously. The optical density (OD) of each well was measured at 572 nm using a Victor^2 V Multilabel Counter plate reader (PerkinElmer, Wellesley, MA, USA). OD measurements were made at 0, 3, 9, 15, and 18 minutes after the assay run began. The absorbance or OD values were subsequently transformed into % platelet aggregation values. Results are expressed as the maximum aggregation response achieved over the 5 time points.

2.21 Agonist induced P-selectin expression assay

Agonist induced platelet activation (P-selectin expression) was assessed using a flow cytometry based assay modified from Nylander *et al* [173]. In a total reaction volume of 100 µl (JNL buffered solution supplemented with 1.8mM CaCl2), 10 µl of PRP or whole blood was incubated with 10 ul of agonist in the presence of 10ul of PE-labelled anti human P-selectin antibody (1.25 µg/ml) or an appropriate isotype control (1.25 µg/ml). All incubations were performed at room temperature for 10 minutes. The reaction was then terminated with 1 ml of JNL buffer prior to analysis by flow cytometry. Samples were analysed using a BD FACS Calibur (Becton Dickinson, Palo Alto, CA, USA) within 1 hour. The instrument was set to measure size (forward scatter, FSC), granularity (side scatter, SSC) and cell flourescence. Using a log FSC vs. log SSC dot plot, a two dimensional analysis gate was drawn around the platelet population, and a fluorescence histogram (log FL2 vs. count) was obtained for 10000 platelet events for each sample. Data was analysed using CellQuest Pro software and expressed as percentage of platelets that were P-selectin positive relative to the isotype control.

2.22 ATP release assay

ATP release by platelets is a measure of dense granule release. Collagen, PAR4-AP, and PAR1-AP induced platelet ATP release was measured using a 96 well plate luminescence based assay that utilises the ATP dependent luciferin - luciferase interaction that generates light. Variable concentrations of agonists (10 µl) were prepared in a flat bottomed 96 well plate through the serial dilution of stock agonist concentrations in JNL buffer (130 mM NaCl, 10 mM sodium citrate, 9 mM $NaHCO_3$, 6 mM D-glucose, and 0.9 mM $MgCl_2$, 0.81 mM KH_2PO_4, and 10 mM Tris, pH 7.4). Washed platelets were prepared and 90 µl aliquots were dispensed into the wells of the plate. The resulting concentrations of agonist were as follows; Collagen (190, 142.5, 71.25, 35.6, 17.8, 8.9, 4.4, and 2.2 µg/ml), PAR 1 activating peptide (20, 10, 5, 2.5, 1.25, 0.6, 0.3, and 0.15 µM), and PAR 4-activating peptide (300,150, 75, 37.5,

18.8, 9.4 µM). A negative control was included in the form of resting platelets. The plate was incubated for 6 minutes with vigorous shaking. 10 ul of Chrono-lume (Chrono-log Corp., Haverton, PA, USA) was added, and the luminescence of each well was read using a Victor^2 V Multilabel Counter plate reader (PerkinElmer, Wellesley, MA, USA). Chrono-Lume is buffered firefly luciferin-luciferase. The amount of light produced through this interaction is directly proportional to the amount of ATP available. Results are expressed as arbitrary luminescence units.

2.23 Annexin V binding assay

Agonist induced phosphatidylserine exposure on platelets was measured by Annexin V binding, using a flow cytometry based assay modified from Nylander et al [173]. In a total reaction volume of 100 ul (HEPES buffered solution; 137 mM NaCl, 2.7 mM KCl, 1 mM MgCl2, 5.6 mM glucose, 1 g/l bovine serum albumin, 20 mM HEPES; pH 7.40, supplemented with 2.5mM $CaCl_{2)}$, 10ul of hirudinised PRP was incubated with 5 ul of FITC Annexin V. Platelets were activated with 10ul of agonists alone or in combination. The agonists used alone or in combination were as follows: (1) 20uM PAR1 activating peptide, 300 µM PAR-4 activating peptide, 50 ng/ml Convulxin, or in combination (4) 20 µM PAR-1 activating peptide + 300 µM PAR-4 activating peptide + 50ng/ml Convulxin. The concentrations of agonist used are known to induce maximal P-selectin expression. All incubations were performed at room temperature for 30 minutes. The reaction was terminated with 1 ml of 3.7% PFA (paraformaldehyde) prior to analysis by flow cytometry. Samples were then analysed using a BD FACS Calibur [Becton Dickinson, Palo Alto, CA, USA] within 1 hour. The instrument was set to measure size (forward scatter, FSC), granularity (side scatter, SSC) and cell flourescence. Using a log FSC vs. log SSC dot plot, a two dimensional analysis gate was drawn around the platelet population, and a flourescence histogram (log FL2 vs. count) was obtained for 10000 platelet events for each sample. Data was analysed using CellQuest Pro software and expressed as percentage of platelets that were positive for annexin v binding relative to resting unactivated platelets.

2.24 Statistics

Results were analysed using GraphPad prism (GraphPad Software Inc., San Diego, CA, USA). All presented data represents the results of 3 independent experiments. Results are expressed as mean ± the standard error of the mean or mean ± standard deviation. The Kolmogorov–Smirnov test was used to assess if data sets were parametric or non parametric. Parametric data was analysed by student t test. Non parametric data was analysed Mann Whitney tests. For paired data, Wilcoxon matched pair test (non parametric data) or paired t test (parametric data) was used. A value of $P \leq 0.05$ was considered statistically significant.

CHAPTER 3: Platelet adhesion and degranulation induce proliferative, pro-angiogenic, and anti-apoptotic signalling in ovarian cancer cells

3.1 Introduction

Ovarian cancer is the 5th leading cause of cancer related deaths in women [174]. It is the most common gynaecologic malignancy and has the highest fatality to case ratio of all gynaecologic malignancies. The poor ratio of survival is the result of late stage diagnoses, most patients are asymptomatic until the disease has metastasised [175]. 90 % of all ovarian cancers are epithelial in nature, arising from the malignant transformation of the pluripotent cells of the celomic epithelium. Epithelial ovarian cancer is predominantly a disease of perimenopausal and postmenopausal women. The peak age of incidence is 60 years. Family history, mutations in BRCA-1 and BRCA-2 genes, infertility, early menarche, and late menopause are established risk factors for ovarian cancer. Oral contraceptive use, tubal ligation, hysterectomy, and multiparty appear to confer protection against ovarian cancer [174, 175].

The spread of ovarian cancer has been considered to occur primarily via invasion into the peritoneum. However, autopsy and imaging studies, as well as evidence for the presence of micrometastases in the bone aspirates of early stage ovarian cancer patients suggest that hematogenous metastasis is more common than previously thought and occurs early in disease progression. There is significant evidence to suggest that platelets are important in the pathology of ovarian cancer. Thrombosis is a frequent complication of ovarian cancer and associated with reduced survival [141, 176, 177]. In a meta-analysis of 40 studies on the incidence of thrombosis in cancer patients, Iodice *et al* found that of all cancers, ovarian cancer was associated with the highest incidence of thrombosis[176]. Thrombocytosis is also a frequent complication of ovarian cancer and associated with poor prognosis [178, 179].

In a recent study using a murine model of ovarian cancer, Stone *et al* showed thrombocytosis associated with ovarian cancer was caused by tumour secreted Il-6 increasing the hepatic synthesis of thrombopoietin leading to increased platelet production[179]. Interestingly, in the same study, the authors showed that high numbers of platelets extravasate out of the circulatory system and infiltrate the ovarian tumour microenvironment. The use of an antiplatelet antibody to induce a 50% reduction in platelet count in ovarian tumour bearing mice was associated with a significant decrease in tumour growth and angiogenesis [179]. This suggests that platelets induce signalling in tumour cells that influence disease progression. However, whether platelet adhesion and degranulation induce tumour cell signalling events that could influence disease progression is not known.

Using ovarian cancer cell lines as a model system, it was hypothesised that platelet interactions with ovarian tumour cells could induce signalling events that could influence disease progression. First, the ability of platelets to adhere to ovarian cancer cells was investigated. Second, the ability of ovarian cancer cells to induce platelet activation and degranulation was assessed. Third, having shown that platelets adhere to and are activated by ovarian cancer cells, gene expression profiles in ovarian cancer cells treated with washed platelets *(representing platelet adhesion)* and platelet releasate *(representing platelet degranulation)* were investigated. The results of the study demonstrate for the first time that platelet adhesion platelet degranulation can induce proliferative, anti-apoptotic, and pro-angiogenic signalling within ovarian cancer cells that could potentially modulate the metastatic potential of tumour cells and influence disease progression *in vivo*.

3.2 Results

3.2.1 Platelets adhere to ovarian cancer cells under static conditions

Firstly, it was assessed whether platelets adhere to ovarian cancer cells under static conditions. Cells were cultured in the wells of 96 well plates until confluent. Calcein AM labelled washed platelets were added and % platelet adhesion was quantified based on the fluorescent detection of labelled adherent platelets. Platelet adhesion to fibrinogen and albumin were used as positive and negative controls (Figure 3.1). Platelet adhesion to A2780 cells (2.4 ± 0.2 %, n = 8), its cisplatin resistant daughter cell line A2780cis (3.0 ± 0.2 %, n = 8) and 59M cells (3.4 ± 0.3 %, n = 8) was significant compared to the negative control albumin (1.0 ± 0.1 %, n = 8). Platelet adhesion to HIO-80 (1.8 ± 0.2 %, n = 8) and SKOV-3 (1.3 ± 0.2 %, n = 8) cells was not significant compared to platelet adhesion to albumin. % adhesion to all 5 cell lines was significantly lower than fibrinogen (5.9 ± 0.4 %, n = 8, $p > 0.01$). Platelet adhesion was also imaged by fluorescence microscopy. Fluorescence microscopy images clearly demonstrate platelet adhesion to the ovarian cancer A2780 and 59M cell lines (Figure 3.1 B and C).

3.2.2 Ovarian cancer cells induce platelet activation and degranulation in a dose dependent manner

The ability of ovarian cancer cells to induce platelet activation and degranulation was assessed by flow cytometry. Increasing concentrations of ovarian cancer cells (0 – 1.5 x 10^6 cells/ml) were added to PRP and platelet activation and degranulation was measured by P-selectin expression. P-selectin is found internally on platelet alpha granules but is translocated to the surface during platelet activation. Ovarian cancer cells induced a dose-dependent increase in platelet P-selectin expression (Figure 3.2). The two metastatic ovarian cancer cell lines, SKOV-3 (1.5 x 10^6 cells/ml, 47 ± 10 %) and 59M (1.5 x 10^6 cells/ml, 51 ± 18 %, n = 6) induced the most significant P-selectin expression. The lowest platelet activation was seen in response to the non-metastatic ovarian cancer cell line A2780 (1.5 x 10^6 cells/ml, 16 ± 5 %, n = 6).

A2780cis demonstrated higher platelet P-selectin expression than its parent cell line A2780 (1.5 x 10^6 cells/ml, 28 ± 12 %, n = 3). The immortalised normal ovarian epithelial cells line HIO-80 also induced platelet P-selectin expression (1.5 x 10^6 cells/ml, 33 ± 8 %, n = 3), but to a lesser extent than the metastatic 59M and SKOV-3 cell lines. Platelet integrin αIIbβ3 activation induced by A2780 and 59M cells (1.5 x 10^6/ml) was also examined (Figure 3.3). Similar to the P-selectin data, 59M cells (19 ± 6 %, n = 6) induce more αIIbβ3 activation than A2780 cells (4 ± 2 %, n = 6).

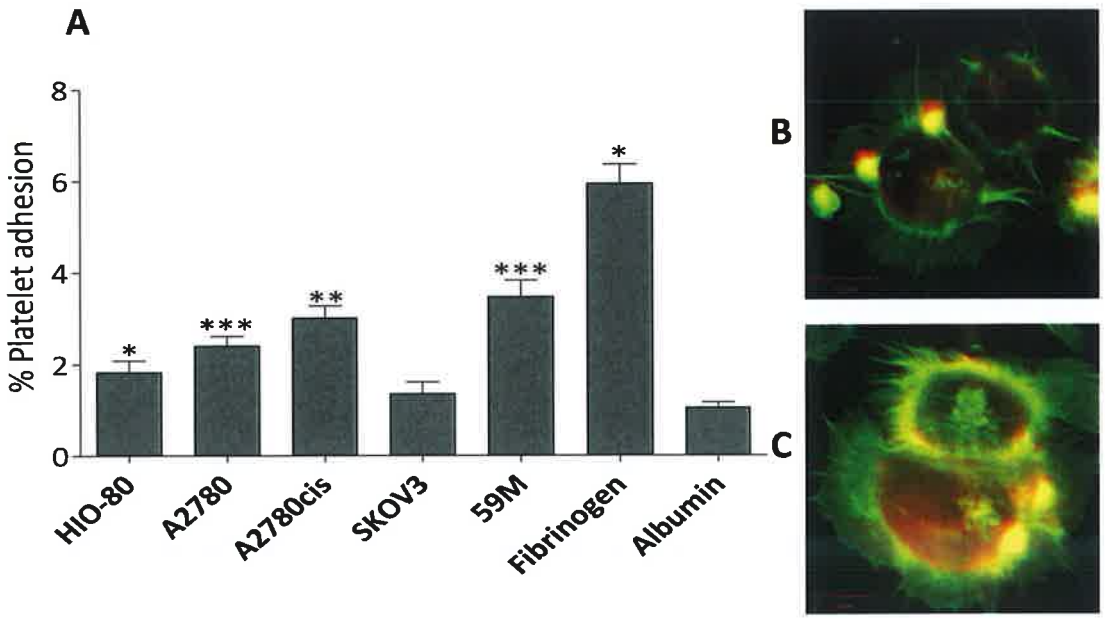

Figure 3.1: Platelet adhesion to ovarian cancer cell lines is heterogeneous under static conditions (n = 8, mean + SEM). (A) Platelet adhesion to ovarian cancer cells was quantified based on the fluorescence detection of labelled platelets. Platelet adhesion to fibrinogen and albumin were used as positive and negative controls. There was significant variation in platelet adhesion across the five ovarian cell lines. To determine if platelet adhesion to ovarian cancer cells was significant, the percentage of platelet adhesion to the 5 cell lines was compared to platelet adhesion to the negative control, albumin. Data was analysed using paired t-test *(HIO-80, A2780, SKOV3, fibrinogen, * = $p \leq 0.05$ vs. albumin)* or wilcoxon matched pair test *(A2780cis, * = $p \leq 0.05$ vs. albumin)*. Platelet adhesion to ovarian cancer cells was imaged by fluorescence microscopy. Ovarian cancer cells and platelets were stained for actin, platelets were stained specifically for CD42a (red/yellow). Images clearly show platelet adhesion to **(B)** A2780 and **(C)** 59M cells under static conditions (representative of n = 3).

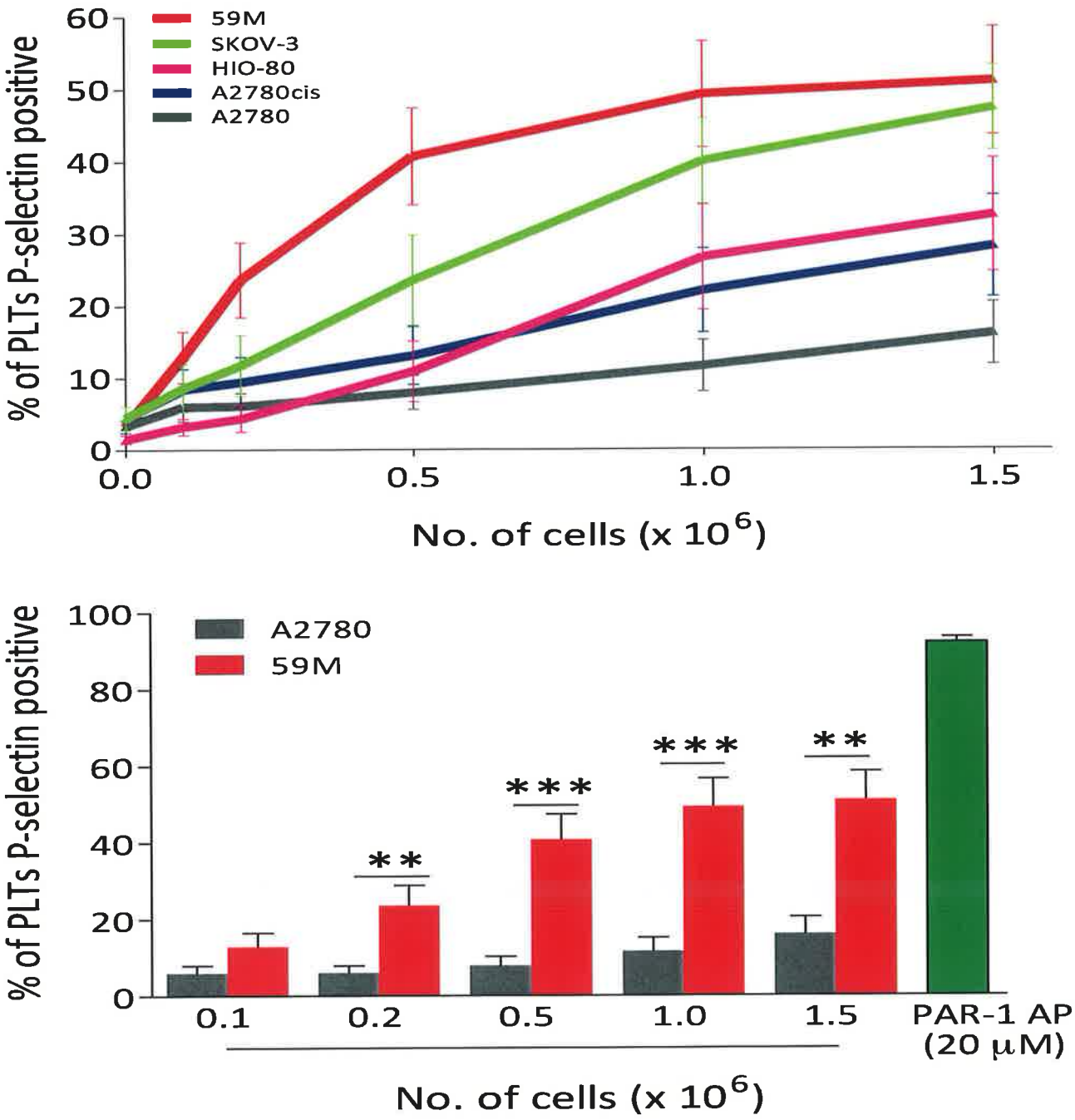

Figure 3.2: Ovarian cancer cell lines induce platelet activation and degranulation in a dose dependent manner (mean ± SEM, n = 3). (A) PRP was incubated with ovarian cancer cells and platelet activation was measured by flow cytometry based on P-selectin expression. Results are expressed as the % of platelets P-selectin positive relative to an isotype control. All ovarian cell lines induced platelet activation in a dose dependent manner. The most significant platelet activation was seen in response to the metastatic ovarian cancer cell lines SKOV-3 and 59M. **(B)** A comparison of platelet activation by 59M and **A2780** cells, the cell lines that induce the greatest and least platelet activation. PAR-1 activating peptide (20 μM) was included as a positive control for platelet activation. There was a significant difference in platelet activation induced by 59M and A2780 cells at multiple concentrations. Data was analysed using wilcoxon matched pair test (*p = ≤ 0.05).

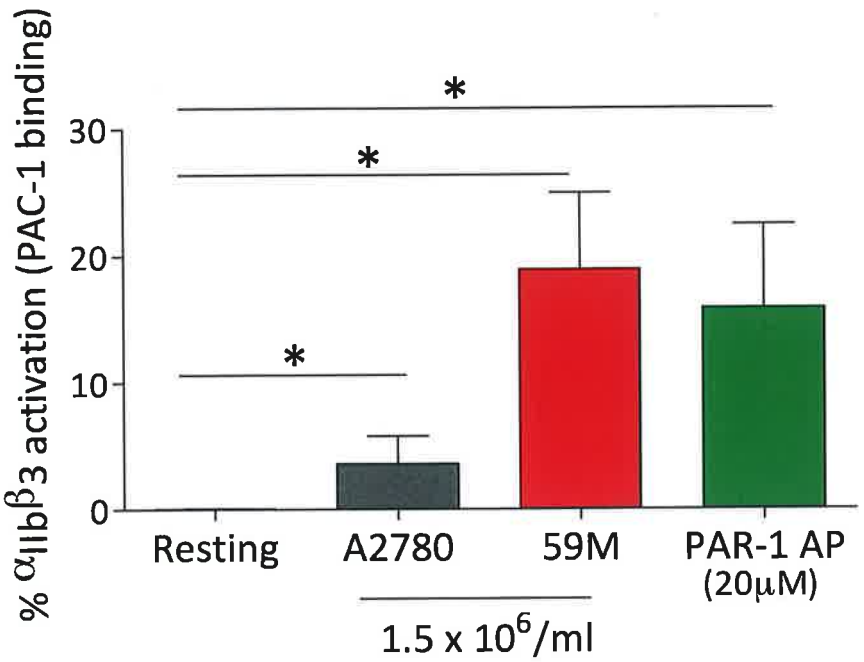

Figure 3.3: A2780 and 59M cells induce platelet integrin αIIβ3 activation in the same pattern as P-selectin expression (mean + SEM, n = 6). PRP was incubated with 59M or A2780 ovarian cancer cells (1.5 x 10^6/ml) and platelet activation was measured by αIIbβ3 activation. PAR-1 activating peptide (20 μM) was included as a positive control for platelet activation. Results are expressed as the % of platelets positive for PAC-1 binding (a marker of αIIbβ3 activation) relative to an isotype control. Similar to the pattern of P-selectin expression, 59M also induce more αIIbβ3 activation than A2780 cells. PAR-1 AP (20 μM), 59M (1.5 x 10^6/ml), A2780 (1.5 x 10^6/ml) treated PRP samples showed significantly more αIIbβ3 activation when compared to resting PRP samples. Data was analysed using wilcoxon matched pair test (*= $p \leq 0.05$).

3.2.3 ADP, P2Y1 receptor, and P2Y12 receptor antagonists inhibit ovarian cancer cell induced platelet activation

Since 59M cells caused the most significant platelet activation, they were used to test the effect of a range of platelet inhibitors on ovarian cancer cell induced platelet activation. 59M ovarian cancer cells (1.5×10^6 cells/ml) were added to PRP pre-treated with inhibitors and platelet activation was measured by flow cytometry (P-selectin expression). The % of platelets P-selectin positive in response to 1.5×10^6/ml of 59M cells was normalised to 0 % inhibition. Antagonists against Thrombin (Hirudin), integrin αIIbβ3 (Reopro and RGDS), COX-1 (aspirin), and calcium (EDTA) had no effect on 59M cell induced platelet activation (n = 3, Figure 3.5). The fact that hirudin had no inhibitory effect suggests that 59M cell induced platelet activation is not dependent on the ability of ovarian cancer cells to activate the coagulation cascade, leading to thrombin generation, and thrombin dependent platelet activation.

Following treatment with the P2Y12 antagonist (cangrelor, 1μM), the P2Y1 antagonist (MRS2179, 10 μM) or the ADP/ATPase (apyrase, 10 units/ml), platelet activation in the presence of 59M ovarian cancer cells was significantly diminished (1 μM Cangrelor - 92 ± 1 % inhibition, $p<0.001$; 10 μM MRS2179 – 71 ± 11% inhibition, $p = 0.01$; 10 units/ml apyrase, 92 ± 4 % inhibition, $p < 0.001$, n = 3) (Figure 3.4a). ADP induced platelet activation was also inhibited by cangrelor, MRS2179, and apyrase, while PAR-1 activating peptide induced platelet activation was not, showing the specificity of these antagonist for the ADP pathway (Figure 3.4 B-C). Interestingly, the cell free supernatant of 59M cells induced comparable platelet activation to 59M cells, suggesting that a soluble factor released by the cells was responsible for platelet activation rather than cell to cell contact dependent interactions (Figure 3.4a).

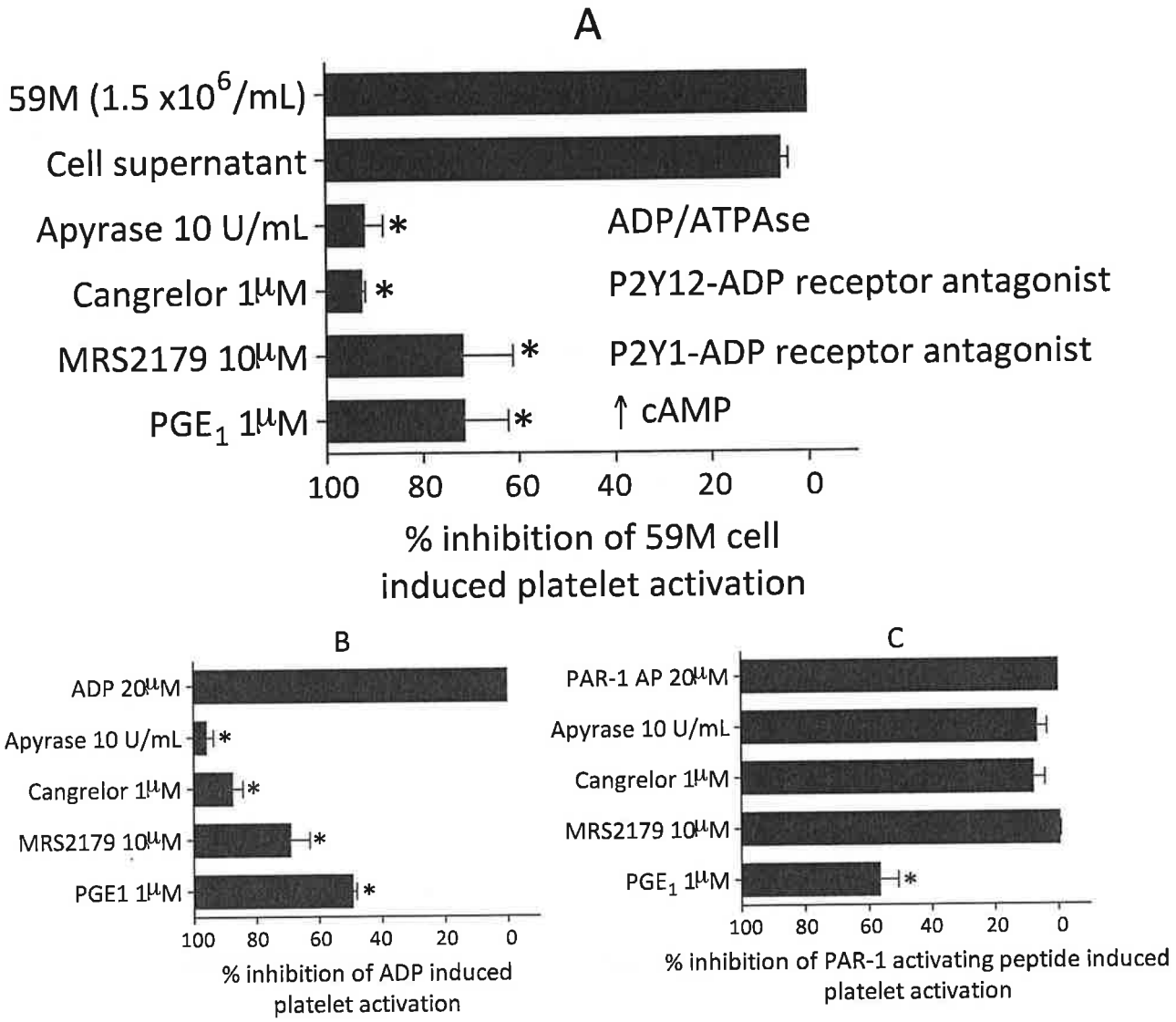

Figure 3.4: 59M tumour cell induced platelet activation is inhibited by cangrelor, MRS2179, and apyrase (mean + SEM, n = 3). (A) 59M cells (1.5 x 10^6/ml) were added to PRP pretreated with inhibitors and platelet activation was measured by flow cytometry (P-selectin expression). The % of platelets P-selectin positive in response to 1.5 x 10^6/ml of 59M cells was normalised to 0% inhibition. 59M cell induced platelet activation is inhibited by antagonists of ADP induced platelet activation. The P2Y12 antagonist cangrelor (1μM), the P2Y1 antagonist MRS2179 (10 μM) and the ADP/ATPase (apyrase, 10 units/ml) all decreased platelet activation by 59M cells (* = $p \leq 0.05$, vs. 59M (1 x 10^6/ml), paired t test). 59M cell free supernatant of 59M cells induced comparable platelet activation to 59M cells, suggesting that a soluble factor released by the cells was inducing platelet activation rather than or as well as cell to cell contact dependent interactions. Figures 3.3B and 3.3C show the results of ADP (20μM) and PAR-1 (20μM) activating peptide induced platelet activation **(B)** Apyrase, cangrelor, and MRS2159 inhibited ADP induced platelet activation but had no effect on **(C)** PAR-1 activating peptide induced platelet activation (* = $p \leq 0.05$, vs. 20 μM ADP/PAR-1 activating peptide, paired t test). This highlights the specificity of cangrelor, MRS2179, and apyrase for the ADP induced platelet activation pathway. Data was analysed using paired t tests.

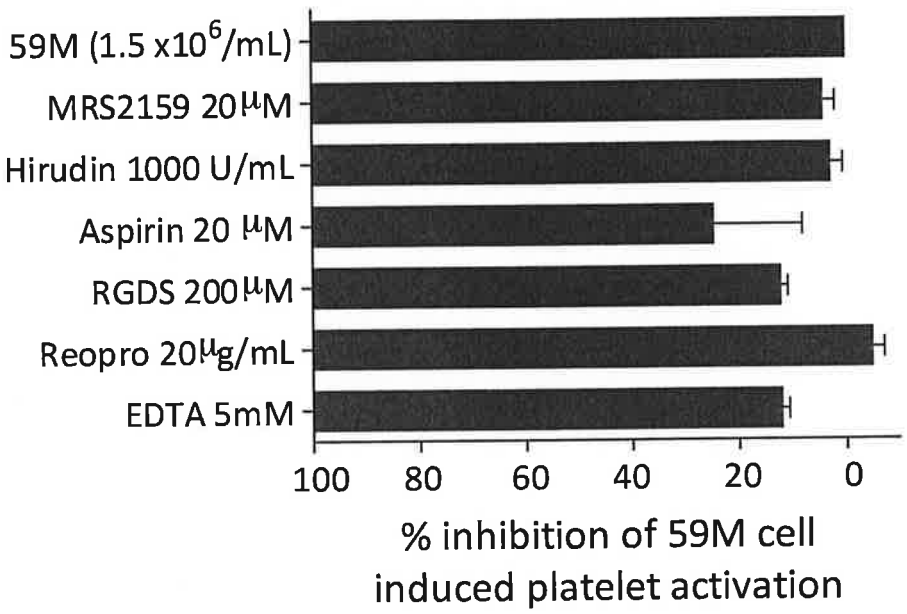

Figure 3.5: 59M tumour cell induced platelet activation is not inhibited by Hirudin, Reopro, RGDS, Aspirin, MRS2159 or EDTA (mean + SEM, n = 3). 59M ovarian cancer cells were added to PRP pre-treated with inhibitors of platelet function. Platelet activation was measured by flow cytometry (P-selectin expression). The % of platelets P-selectin positive in response to 1.5 x 10^6/ml of 59M cells was normalised to 0% inhibition. Inhibitors of the P2X1 receptor (MRS2159), thrombin (hirudin), COX-1 (aspirin), the αIIbβ3 receptor (Reopro and RGDS), or Ca^{2+} (EDTA) had no effect on 59M tumour cell induced platelet activation. Data was analysed using paired t tests.

3.2.4 Ovarian cancer cells potentiate PAR-1 activating peptide, PAR 4 activating peptide, and arachidonic acid induced platelet activation in a dose dependent manner

Since platelet activation and degranulation in vivo is a complex process involving multiple agonists, the ability of ovarian cancer cells to modulate soluble agonist *(PAR-1 activating peptide, PAR-4 activating peptide, arachidonic acid, ADP, epinephrine, and collagen)* induced platelet activation was investigated. To assess this, platelet rich plasma was incubated with a low concentration of soluble agonist, a low 59M cancer cell concentration, or both in combination. Platelet activation was then measured by P-selectin expression. Soluble agonist and 59M cell concentrations that gave less than or equal to 20 percent platelet P-selectin expression were used. At low cellular concentrations (0.5 - 1 x 10^5 cells/ml), 59M ovarian cancer cells potentiated PAR-1 (PAR-1 activating peptide), PAR-4 (PAR-4 activating peptide) and TxA_2 receptor (arachidonic acid) mediated platelet activation (Figure 3.5) but had no effect on ADP, epinephrine, or collagen induced platelet activation (Figure 3.6). For example, in response to 1 µM PAR-1 activating peptide and 1 x 10^5 59M cells/ml, platelet P-selectin expression was 44 ± 12 % compared to 4 ± 2 % in response to 1 x 10^5 59M cells/ml alone, and 5 ± 1 % in response to 1 µM PAR-1 activating peptide alone (n = 3, Figure 3.6). Conversely, in response to 1 µM ADP and 1 x 10^5 59M cells/ml, platelet P-selectin expression was 17 ± 6 % compared to 2 ± 1 % in response to 1 x 10^5 59M cells/ml alone and 15 ± 5 % in response to 1 µM ADP alone (n = 3, Figure 3.7). Similar results were also seen in response to A2780 cells but higher A2780 cell concentrations were required to induce a similar effect (data not shown). This data suggests a synergistic relationship between PAR-1, PAR-4, and TxA2 receptor mediated platelet activation and A2780/59M induced platelet activation. In line with the inhibitory effect of antagonists of the ADP pathway on 59M cell induced platelet activation, this synergistic interaction was also inhibited by the P2Y12 receptor antagonist cangrelor (data not shown).

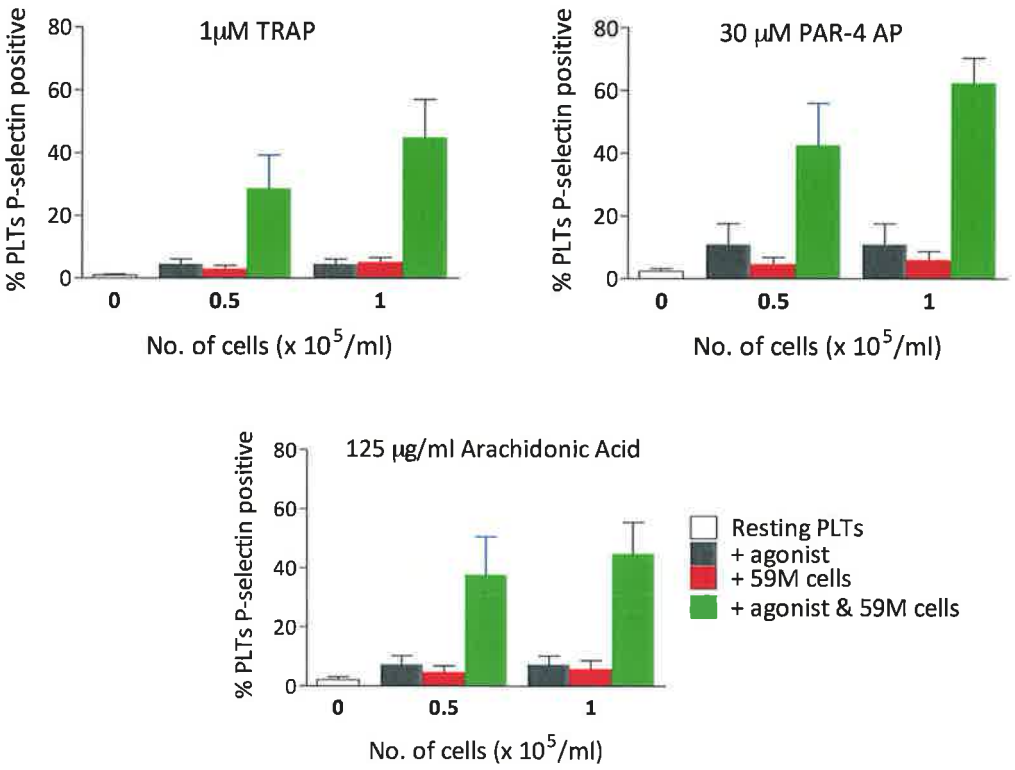

Figure 3.6: 59M cells potentiate PAR-1, PAR-4, and TxA_2 receptor mediated platelet activation (mean + SEM, n = 3). Platelet rich plasma was incubated with low concentration of **soluble agonists**, **59M cells**, or **both in combination**. Platelet activation was measured by P-selectin expression. At low cell concentrations, 59M (0.5 or 1 x 10^5/ml) cells significantly potentiated PAR-1 activating peptide (1 μM), PAR4 activating peptide (30 μM), and arachidonic acid (125 μg/ml) induced platelet activation. This suggests a synergistic relationship between PAR-1, PAR-4, and TxA2 receptor mediated platelet activation and 59M induced platelet activation. Similar results are seen with A2780 cells but higher concentrations of cells are required (1 or 5 x 10^5 cells/ml for required to elicit a similar effect, data not shown).

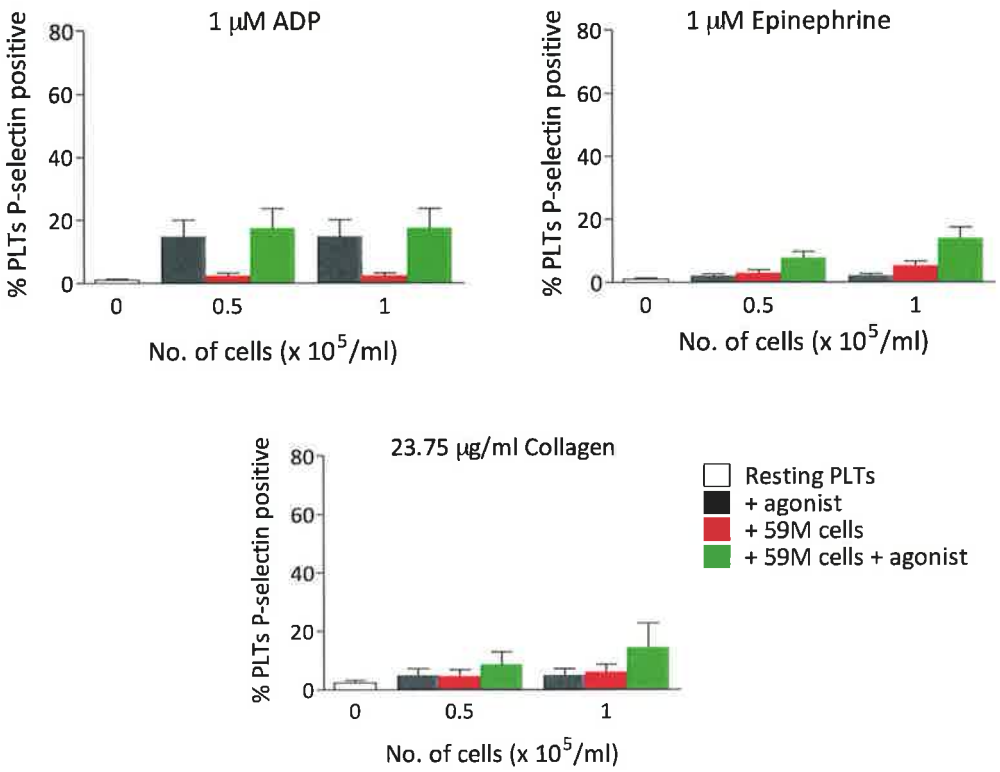

Figure 3.7: 59M cells have no effect on ADP, collagen, and epinephrine induced platelet activation (mean + SEM, n = 3). Platelet rich plasma was incubated with low concentration of **soluble agonists**, 59M cells, or both in combination. Platelet activation was measured by P-selectin expression. In comparison to PAR-1, PAR-4, and TxA2 receptor mediated platelet activation, 59M cells (0.5 or 1 x 10^5/ml) did not potentiate ADP (1 μM), epinephrine (1 μM) or collagen (23.75 μg/ml) induced platelet activation. Similar results were seen with A2780 cells (data not shown).

3.2.5 Platelet adhesion and degranulation induce proangiogenic and anti apoptotic signalling in 59M cancer cells

Having shown that ovarian cancer cells support platelet adhesion and induce platelet degranulation, it was next determined how platelet adhesion and degranulation affect ovarian cancer cells. In collaboration with Prof. John O' Leary's group at TCD, gene expression changes at the transcriptome level in the 5 ovarian cancer cell lines (HIO-80, SKOV-3, A2780, A2780cis, and 59M cells) following treatment with either washed platelets *(adhesion)* or platelet releasate *(degranulation)* were explored. In the collaboration, washed platelets and platelet releasate were prepared at RCSI while the gene expression analysis was performed at TCD. The results were published in the PLoS One Journal in 2011[180].

Here, the results obtained for the 59M cell line will be presented. 59M cells were treated with washed platelets (2.5 x 10^6/ml) or an equivalent volume of platelet releasate. As it is well established that high concentrations of platelets inhibit growth in a range of human cancer cell lines [181], this concentration of platelets was chosen as it was the highest concentration of platelets that did not inhibit tumour cell growth, as measured by MTT proliferation assay[180]. The cells were treated with platelets or releasate for 6 hours, RNA was isolated and analysed using the Affymetrix Human Exon 1.0 St Array and the Fluidigm qPCR 48 x 48 dynamic array [180]. All experiments were performed in triplicate. The Affymetrix Human Exon 1.0 St Array is a whole transcript array for profiling the expression level of thousands of genes simultaneously. It uses 25-mer oligonecleotide probes to measure the abundance of mRNA transcripts. The array contains multiple probes per exon and up to 40 probes per gene. It is one of the most widely used commercial platforms for gene expression profiling [182]. In the array, β actin and GAPDH are used as endogenous controls. Following treatment, genes that showed a 1.5 fold increased or decreased expression relative to resting untreated cells were considered significant. Gene expression was validated using Fluidigm's high throughput qPCR 48.48 dynamic array system. Genes displaying the most significant fold changes in

addition to genes involved in biologically relevant pathways were selected for validation.

Using the Affymetrix array, 59M cells showed significant (1.5 -1.9 fold changes) changes in the expression of a number of genes following treatment with washed platelets (5 genes) or platelet releasate (13 genes) (Table 3.1). These included genes linked with anti-apoptotic signalling (CCL2), pro angiogenic signalling (PDGFB), immune suppression (CD274 and PDL1), and cell adhesion and migration (ICAM-1). Genes showing the most significant change in expression in 59M cells following treatment with platelets or platelet releasate (CCL2, TRAF1, HBEGF, CD274, TNFAIP2, BIRC3, ICAM1, SH2B3, and DSN1) were also analysed using the Fluidigm platform. Results from the Fluidigm array were comparable to results obtained from Affymetrix array, but slightly increased fold changes in gene expression were seen with the Fluidigm platform (Table 3.1). In comparison to 59M cells, no genes displayed significantly altered expression profiles in A2780 cells following treatment with washed platelets or platelet releasate [180].

Table 3.1: Affymetrix array data for 59M cells genes showing altered expression following treatment with platelets or platelet releasate. Expression changes for genes highlighted in red were also verified using the Fluidigm array platform.

Treatment	Gene	Affymetrix		Fluidigm	
		Fold change	p-value	Fold change	p-value
Washed Platelets	TRAF1	1.9	< 0.01	2.87	< 0.01
	CCL2	1.78	< 0.05	2.67	< 0.01
	TNFAIP2	1.63	< 0.01	1.86	< 0.01
	PDGFB	1.5	< 0.05	2.45	< 0.01
	DSN1	-1.6	< 0.05	- 1.09	< 0.01
Platelet Releasate	CCL2	1.94	< 0.05	2.83	< 0.01
	TRAF1	1.92	< 0.01	3.5	< 0.01
	HBEGF	1.67	< 0.01	2.01	< 0.01
	CD274	1.64	< 0.05	2.09	< 0.01
	ANKRD1	1.60	< 0.01		
	TNFAIP2	1.59	< 0.01	2.51	< 0.01
	BIRC3	1.58	< 0.05	1.8	< 0.01
	ICAM1	1.57	< 0.01		
	IRAK2	1.56	< 0.01		
	SH2B3	1.53	< 0.01	2.2	< 0.01
	CSF2	1.52	< 0.05		
	GCH1	1.52	< 0.05		
	HIVEPI	1.51	< 0.05		

3.3 Discussion

There is conclusive evidence that platelets are important in the pathology of cancer. However, it was not currently known if platelets could induce signalling in tumour cells that could promote disease progression. Rather, the role of platelets in the pathology of cancer has focussed on their ability to modulate the activity of other cell types (e.g) NK cells, and how this protects tumour cells from immune surveillance. Using ovarian cancer cell lines as a model system, it was hypothesised that platelet interactions with ovarian tumour cells could induce signalling events in tumour cells that could influence disease progression. In this study, we demonstrate for the first time that platelet adhesion and degranulation induce anti-apoptotic and pro-angiogenic signalling in ovarian cancer cells that could modulate their metastatic potential *in vivo*

First, platelet adhesion to a range of ovarian cancer cells was assessed. Adhesion across 5 cells lines was extremely heterogeneous with significant platelet adhesion to the 59M, A2780, HIO-80 and A2780cis cell lines, while adhesion to the SKOV-3 cell line was not significant compared to the negative control albumin. The adhesion of platelets to all ovarian cancer cell lines was significantly less than to fibrinogen, suggesting that the adhesion is mediated by either a lower copy number receptor-ligand interaction, or a lower affinity interaction than platelet integrin αIIbβ3 mediated adhesion to fibrinogen (Figure 3.1). While this study utilised static adhesion assays, ovarian cancer cells can also support platelet adhesion under dynamic conditions, as evidence by Figure 4.2.

Second, the ability of ovarian cancer cells to induce platelet activation and degranulation was studied, using P-selectin as a marker of platelet activation. Ovarian cancer cells induce platelet activation and degranulation in a dose dependent manner with the most significant platelet degranulation seen in response to the metastatic 59M and SKOV-3 ovarian cancer cell lines (Figure 3.2). It is commonly reported that the ability of tumour cells to induce platelet activation

correlates with their metastatic potential. In a frequently cited article, Pearlstein *et al* showed a correlation between metastatic potential and the ability of cancer cells to induce platelet activation [183]. In the study, the authors subcutaneously implanted PW20 rat renal sarcoma cells in immunocompetent rats. Cells that metastasised were subsequently isolated and cultured. These cells were then implanted in another rat and cells that metastasised were again isolated. This procedure was used to generate 10 clones of the successive generations of the PW20 cell line that had increasing metastatic potential. The ability of the 10 PW20 clones to induce platelet aggregation was directly correlated with their metastatic potential. However, Estrada and Nicholson failed to show the same correlation in a similar rat mammary adenocarcinoma model [184], suggesting that the ability of cancer cells to induce platelet activation is extremely heterogeneous and unrelated to metastatic potential.

Following treatment with cangrelor (P2Y12 antagonist), MRS2179 (P2Y1 antagonist), or apyrase (an ADP/ATPase) platelet activation in the presence of 59M ovarian cancer cells was greatly diminished, suggesting a P2Y12/P2Y1 dependent mechanism of activation (Figure 3.4a). Consistent with the literature, other studies have also demonstrated ADP dependent platelet activation induced by cancer cells[185]. Notably, Uluckan *et al* have shown that APT102 (an ADPase) and aspirin in combination inhibited B16 melanoma cell induced aggregation and decreased metastasis in a murine B16 melanoma model of bone metastasis [63]. The mechanism of tumour cell induced platelet activation could be physiologically relevant. Upon degranulation, platelets release the contents of their storage granules. Platelet granules are rich sources of growth and angiogenic factors, such as platelet derived growth factor (PDGF) and vascular endothelial growth factor (VEGF) that could potentially promote tumour growth and metastasis *in vivo*. However, platelet granules are also rich sources of anti-growth and anti-angiogenic factors, such as endostatin, that could potentially inhibit tumour growth and metastasis *in vivo*.

Platelets differentially package angiogenic regulatory proteins in separate alpha granules that are subject to differential release depending on the platelet agonist [186].

Platelet activation via the PAR-1 receptor causes the release of the pro-angiogenic factor VEGF, but activation via the PAR-4 receptor causes the release of the anti-angiogenic factor endostatin. ADP induced platelet activation has been also shown to induce differential granule release, causing the release of VEGF but not endostatin *in vitro* [187, 188]. This indicates a potential mechanism by which ovarian cancer cells could induce the release of pro-angiogenic but not anti-angiogenic factors by platelets *in vivo*. Interestingly, MCF-7 breast cancer cells have also been shown to induce ADP dependent platelet activation resulting in the release of VEGF but not endostatin [188].

Platelet activation and degranulation *in vivo* is a complex process involving multiple agonists. Therefore, we examined how ovarian cancer cells modulate soluble agonist induced platelet activation. At low cellular concentrations, 59M cells potentiated PAR-1 activating peptide, PAR-4 activating peptide, and arachidonic acid induced platelet activation (Figure 3.6), but not ADP, epinephrine, or collagen induced platelet activation (Figure 3.7). This suggests a synergistic relationship between PAR-1/PAR-4/TxA2 receptor mediated activation and the mode of 59M ovarian cancer cell induced activation. This synergistic interaction could be physiologically relevant *in vivo*. Thrombin is the physiological ligand for PAR-1 and PAR-4 receptors, and its generation is critical for coagulation. The expression of thrombin has been identified in ovarian cancer tissue [189] and is known to potentiate invasion in an *in vitro* model of ovarian cancer [190]. Holmes *et al* have also shown that PAR-1 activating peptide treated platelets increase ovarian cancer cell invasion [191]. Prostaglandin synthesis is also increased in ovarian cancer, with a trend towards higher fold increases in pro-aggregatory TxA2 levels compared to anti-aggregatory PGI_2 levels [192].

Third, having established that ovarian cancer cells support platelet adhesion and induce platelet degranulation (release of granule content), the effect of platelet adhesion and platelet degranulation on ovarian cancer cells was investigated. In collaboration with TCD, we assessed the gene expression profile of 59M ovarian cancer cells following treatment with platelets or platelet releasate by microarray analysis. 59M cells showed significant increases in the expression of a number of

genes when treated with platelets (5 genes) or platelet releasate (13 genes) (Table 3.1). These included genes linked with anti-apoptotic signalling, pro-ro angiogenic signalling, immune suppression, and cell adhesion and migration.

For example, expression of the CCL2 gene was significantly upregulated in 59M cells following treatment with platelets and platelet releasate. CCL2 (CC chemokine ligand) also called MCP-1 (monocyte chemo attractant protein -1) is a member of the CC chemokine family and is known to regulate the recruitment of immune cells, such as monocytes to sites of inflammation. The CCL2 gene is inducible by platelet derived growth factor (PDGF) mediated signalling. PDGF is stored in high concentrations within platelet granules and released upon activation, suggesting a mechanism by which platelets could induce increased CCL2 expression[193]. There is abundant evidence to suggest CCL2 may play a role in the progression of solid tumours. The expression of CCL2 is upregulated in a variety of cancer types including ovarian cancer[193]. CCL2 is known to have anti-apoptotic properties. It has been shown to protect breast cancer cells from serum starvation induced apoptosis, prostate cancer cells from chemotherapy induced cytotoxicity [194] and cardiac myocytes from hypoxia induced cell death [195]. It has also been shown to induce cell proliferation in prostate cancer cells *in vitro*[196]. CCL2 has also been shown to have proangiogenic properties. Human umbilical vein endothelial cells (HUVECs) respond to CCL2 by forming vessel sprouts *in vitro* [197]. Hence, the increased expression of CCL2 represents proliferative, anti-apoptotic, and pro-angiogenic signalling in 59M cells mediated by platelet adhesion and degranulation. This could potentially promote growth, angiogenesis, and metastasis *in vivo*.

In conclusion, these results clearly demonstrate for the first time that platelet can induce signalling in tumour cells that could potentially influence disease progression. More recently, Labelle *et al* have also shown platelet induced signalling in breast cancer cells. The authors demonstrated that platelet derived TGFβ activated the NFκB pathway leading to the increased expression of a number of genes, notably CCL2. These signalling events were shown to increase the metastaic potential of platelet treated Ep5 breast cancer cells in an experimental model of pulmonary

metastasis [90]. Also, more specifically in relation to ovarian cancer, Stone *et al* have shown that high numbers of platelets extravasate out of the circulatory system and infiltrate the ovarian tumour microenvironment in a murine model of ovarian cancer. The use of an antiplatelet antibody to induce a 50% reduction in platelet count in ovarian tumour bearing mice was associated with a significant decrease in tumour growth and angiogenesis [179]. This suggests that platelets induce signalling in tumour cells that influence disease progression in ovarian cancer. Consistent with the finding of Stone *et al* and Labelle *et al*, the results of this demonstrate that interactions with platelets, specifically platelet adhesion and granule release result in a pro-survival, pro-angiogenic signal for the ovarian cancer cells that could potentially modulate their metastatic potential *in vivo*.

CHAPTER 4: Living in shear - platelet protection in the circulation

4.1 Introduction

Metastasis is the spread of malignant cells from the primary tumour site to distant organs via the lymphatic or haematogenous circulatory system. It is responsible for 90 % of all cancer related deaths [1]. Of all the cells released from primary tumour into the circulation, estimated at 2 - 4 x 10^6 per gram of primary tumour tissue, only 0.01 % of these cells will establish metastatic foci [198]. During bloodborne metastasis, it is commonly thought that the harsh blood environment owing to immune surveillance and shear forces is responsible for the destruction of most circulating cancer cells [199, 200]. This hypothesis is supported by the literature. In animal models, intravenously injected cancer cells undergo apoptosis within two hours of entering the circulation [201]. In a case study of a patient with advanced metastatic breast cancer, of the 659 circulating cancer cells isolated from a 10ml sample of blood, 32 % of cells were in early stage apoptosis, while 35 % were in late stage apoptosis[202]. Despite the harsh environment, viable cancer cells have been isolated from the blood of patients with most types of epithelial cancer [203] suggesting that cancer cells have mechanisms of overcoming immune surveillance and shear forces.

Shear stress is a biomechanical force generated by blood flow. In a blood vessel, flowing blood has a parabolic flow pattern. The flow is fastest in the centre of the vessel and slowest at the vessel wall (Figure 5.1). This creates infinite layers of blood flowing at different speeds relative to one another. For any given layer of blood, one parallel layer of blood is moving faster, the other parallel layer is moving slower. Shear stress is the frictional drag force generated between parallel layers flowing over one another [204]. It is measured in force per unit area (dyn/cm^2) and is a function of the shear or flow rate (inverse seconds, s^{-1}), the vessel radius, and the viscosity of blood (centipascals, cP)[205]. Within the circulation, the arterial and venous vascular trees are characterised by different levels of shear stress. On the venous side, shear

stress ranges from 1 - 4 dyn/cm^2, corresponding to a shear rate of 5 – 200 s^{-1} and blood viscosity of approximately 3-5 cP. On the arterial side, shear stress ranges from 4-30 dyn/cm^2, corresponding to a shear rate of 200-1500s^{-1} and blood viscosity of 3-4 cP [206].

Shear forces are known to modulate cellular function and are important in the pathology of certain disease states, including atherosclerosis [205, 207]. A number of studies also suggest that shear stress may be an important rate limiting factor in metastasis. For example, exposure of adherent glioma cells to low shear stress (0.1-0.6 dynes/cm^2) decreases cell invasion *in vitro*[208]. Further, intestinal cancer cells exposed to arterial shear stress values (12 dyn/cm^2) undergo cell cycle arrest[209]. Cancer cells have been shown to be susceptible to shear stress induced mechanical damage in a cone and plate viscometer *in vitro* [210]. In animal models, intravenously injected cancer cells quickly become apoptotic *in vivo*[201]. The negative effect of shear stress on metastasis is most clearly demonstrated in the study of Wyckoff *et al* [211]. Using fluorescently labelled implanted cancer cells, the authors successfully imaged the intravastion of cancer cells into the circulatory system *in vivo*. Upon intravastion into a blood vessel, many cells underwent significant membrane fragmentation with the most significant degree of fragmentation seen in non metastatic cells [211].

As well as the beneficial effect of platelet cloaking in the protection of cancer cells from immune surveillance[59, 79, 85, 86, 89], it is frequently suggested that platelet cloaking may also protect cancer cells from shear induced damage [74, 90, 163]. However, there is currently no evidence to support or oppose this hypothesis. In this study, the aim was to test the hypothesis that platelet cloaking can protect cancer cells from shear stress induced damage. It was hypothesised that platelets through cloaking, would act as a physical barrier to shear stress and prevent shear induced damage in to cancer cells. To achieve this, we established an *in vitro* model of shear induced damage.

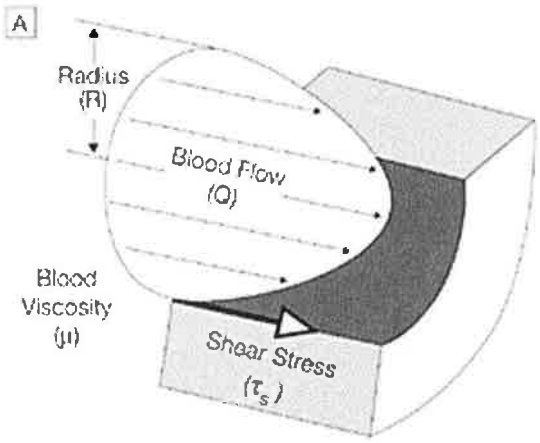

Figure 4.1: Haemodynamic Shear Stress. Cross sectional schematic of a blood vessel showing the parabolic flow pattern of blood. In a vessel, blood moves fastest in the centre and slowest at the vessel wall. This creates infinite layers of blood flowing at different speeds relative to one another. Hence, for any given layer, one parallel layer is moving faster, one is moving slower. Shear stress is the frictional drag force occurring as a result of these layers moving over one another, and is a function of the vessel radius (R), shear rate (Q), and blood viscosity (μ). Adapted from Malek *et al* [204].

In the model, A2780 cells were exposed to shear stress. The A2780 cell line was chosen since it supports platelet adhesion but (Figure 3.1, Figure 4.2) fails to induce significant platelet activation *(P-selectin expression or αIIbβ3 activation)* in suspension (Figure 3.2 & 3.3). As such, the use of the A2780 cell line allows the assessment of the direct effect of platelet cloaking (adhesion) on shear induced damage in cancer cells in the absence of the confounding effect of granule release. The cone and plate viscometer was used to expose suspensions of A2780 cells to shear stress. This machine is advantageous for this type of study for two reasons. Firstly, while the machine is quite laborious to use, it allows suspensions of cells to be exposed to prolonged periods of shear stress (minutes to hours) that are not possible with flow chamber based assays. *In vivo*, tumour cells are found in the circulation up to two hours post intravenous injection, suggesting that prolonged exposure to shear forces is physiologically relevant to circulating tumour cells. Secondly, samples can be easily collected from the cone and plate viscometer and subjected to post treatment analysis, another factor made difficult by the use of flow chamber based assays.

In the study, suspensions of A2780 cells were exposed to venous ($200s^{-1}$, ~ 1.5 dyn/cm^2) and arterial ($1500\ s^{-1}$, ~ 12 dyn/cm^2) shear rates in the presence or absence of platelets and lactate dehydrogenase (LDH) release was used to assess mechanical damage to the cell membrane. First, A2780 cells were shown to support platelet adhesion at venous and arterial shear rates. Second, venous and arterial shear rates were shown to cause membrane damage in A2780 cells, but not platelets. Third, platelets were shown for the first time to confer protection against shear induced damage in cancer cells under physiologically relevant conditions. This protective effect was most evident at the arterial shear rate of $1500s^{-1}$.

4.2 Results

4.2.1 Platelets adhere to A2780 cancer cells under venous and arterial shear rates

In order to determine if platelet cloaking could protect cancer cells from blood flow induced shear damage, a cancer cell line with specific characteristics had to be identified. It had to support platelet cloaking (platelet adhesion) under shear conditions and be susceptible to shear stress induced damage. Based on these 2 characteristics, the A2780 ovarian cancer cell line A2780 was chosen.

Platelet adhesion to A2780 cells was assessed under venous and arterial shear rates. A2780 cells (1 x 10^6/ml) were incubated with a low (50 x 10^6/ml) or high (200 x 10^6/ml) concentration of platelets, resulting in a ratio of 1:50 and 1:200 (A2780 cell: platelets). These samples were exposed to venous ($200s^{-1}$) and arterial ($1500s^{-1}$) shear rates in a cone and plate viscometer for one minute. Preliminary experiments showed platelet adhesion to be maximal at one minute. Platelet adhesion to A2780 cells was quantified using a flow cytometry assay based on the detection of the platelet specific marker CD42b (GPIb alpha) on the surface of A2780 cells following platelet adhesion.

Platelets were found to adhere to A2780 cells under static and shear conditions. The percentage of platelet adhesion (i.e.) the number of A2780 cells positive for CD42b expression, increased slightly as the shear rate increased. At ratio of 1:50, the percentage of platelet adhesion, (i.e.) the number of A2780 cells positive for CD42b expression, was 48 ± 6 % at $0s^{-1}$, 72 ± 2 % at $200s^{-1}$, and 65 ± 6% at $1500s^{-1}$ (n = 3, Figure 4.2a). Platelet adhesion was higher at the 1:200 ratio compared to the 1:50 ratio. At the ratio of 1:200, the percentage of platelet adhesion was 78 ± 5 % at $0s^{-1}$, 88 ± 2 % at $200s^{-1}$, and 81 ± 1 % at $1500s^{-1}$ (n = 3, Figure 4.2c). By comparing the mean fluorescence intensity (MFI) of CD42b staining on a single platelet to the MFI value of CD42b staining on A2780 cells with adherent platelets, it is possible to

estimate the number of platelets that adhere per A2780 cell. At a ratio of 1:50, approximately 3 - 4 platelets were attached per A2780 cells at all shear rates (0-1500s^{-1}, Figure 4.2b). At a ratio of 1:200, approximately 8 – 11 platelets were attached per A2780 cells at all shear rates (Figure 4.2d). Platelet adhesion to A2780 cells under venous shear conditions was also imaged by fluorescence microscopy. Images clearly demonstrate platelet adhesion to A2780 cells at 200s^{-1} (Figure 4.2 e & f).

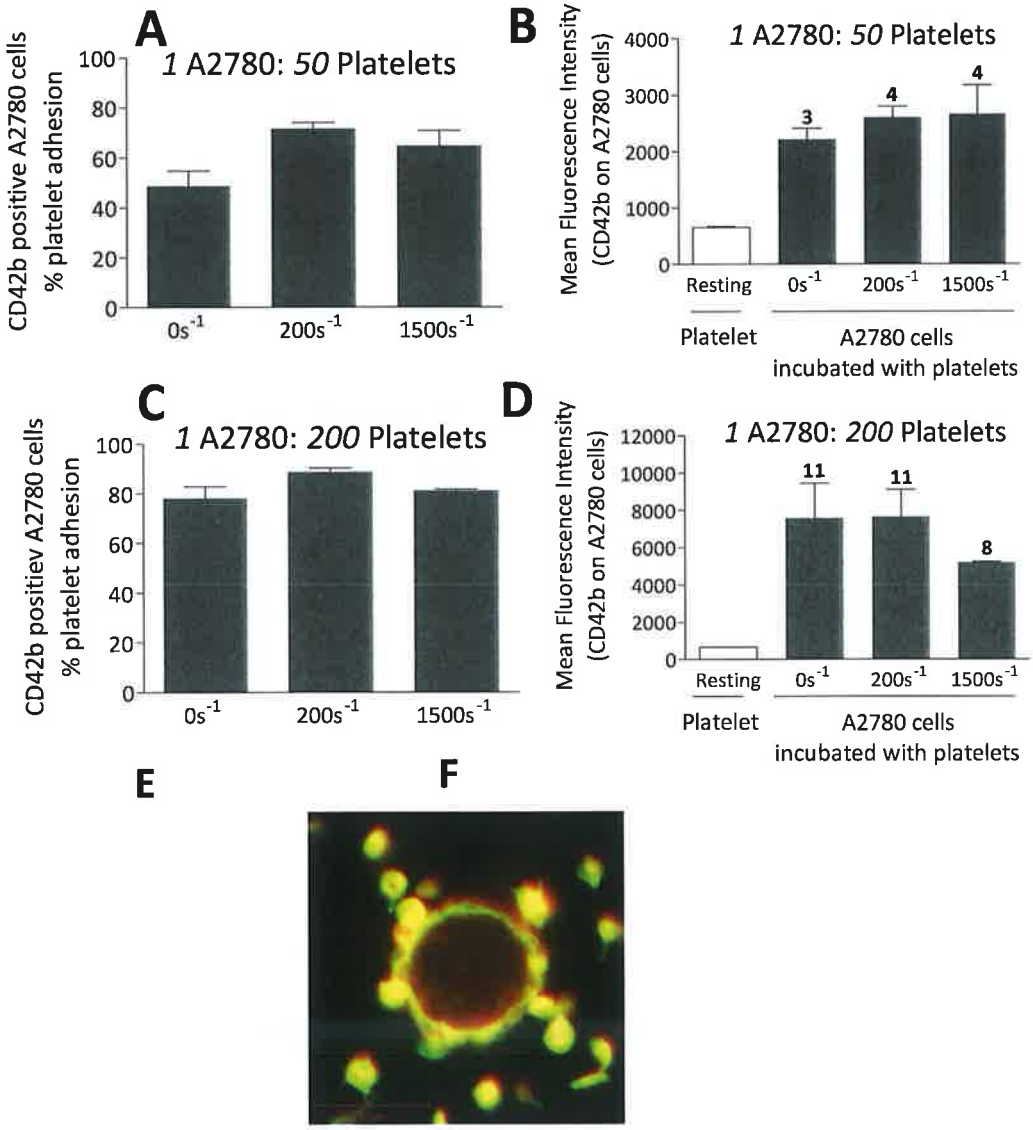

Figure 4.2: Platelets adhere to A2780 cancer cells at venous and arterial shear rates (n = 3, mean ± SEM). A2780 cells (1 x 10^6/ml) were incubated with a low (50 x 10^6/ml) or high (200 x 10^6/ml) concentration of platelets, resulting in a ratio of 1:50 and 1:200 (A2780 cell: platelets). These suspensions were exposed to venous ($200s^{-1}$) and arterial ($1500s^{-1}$) shear rates. Platelet adhesion was quantified by flow cytometry based on the detection of the platelet specific marker CD42b on the surface of A2780 cells. **Figure 4.1 A** (1:50) **C** (1:200) show the amount of A2780 cells positive for the platelet specific marker CD42b following co incubation at a range of shear rates. By comparing the mean fluorescence intensity (MFI) of CD42b staining on a single platelet to the MFI value of CD42b staining on A2780 cells with adherent platelets, it is possible to estimate the number of platelets that adhere per A2780 cell. **Figure 4.1 B** (1:50) and **D** (1:200) show the relative intensity of CD42b staining on A2780 cells following platelet adhesion relative to a single resting platelet (white).The numbers above the bars indicate the number of adherent platelets per A2780 cell. **Figure 4.1 E** and **F** are fluorescent microscopy images of platelets adhering to A2780 cells at $200s^{-1}$. Platelets and A2780 cells were stained with Alex flour 488 phalloidin, and platelets were stained specifically for CD42a (red/yellow).

4.2.2 Determining the optimal cell concentration for LDH release

It was next established whether A2780 cells are susceptible to membrane damage under venous and arterial shear rates. Lactate dehydrogenase (LDH) release was used to assess membrane damage, as measured by the TOX-7 cytotoxicity kit. The use of LDH release allowed the assessment of mechanical damage that would likely occur on a shorter time scale than biochemical damage (e.g.) induction of apoptosis. Based on the TOX-7 kit guidelines, the optimal concentration of cells for LDH release must be determined for each cell line. This is the concentration of cells that gives the biggest difference in LDH levels when comparing corresponding resting and lysed samples. The optimal concentration of A2780 cells for LDH release was determined to be 1×10^6/ml. In additional experiments, LDH release by platelets was also studied. The optimal concentration for platelets was 2.5×10^8/ml. At these two concentrations, platelets and A2780 cells release comparable levels of LDH upon lysis (Figure 4.3). These concentrations were used in subsequent experiments to assess LDH release under shear conditions.

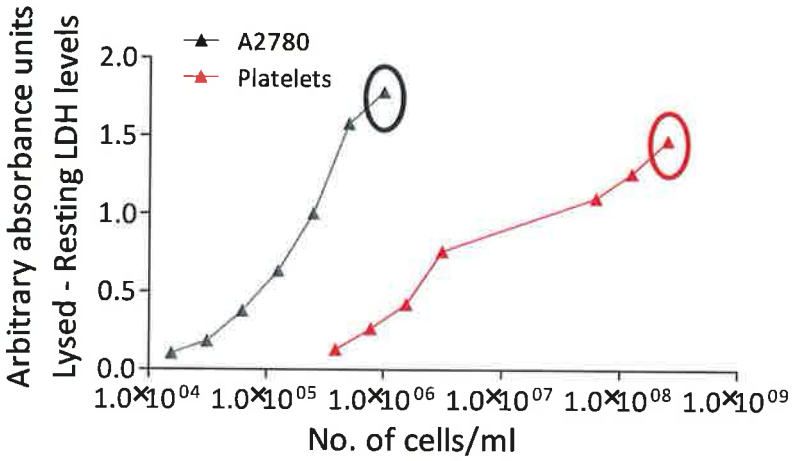

Figure 4.3: Determining the optimal concentration of cells for measuring LDH release. Based on the TOX-7 kit guidelines, the optimal concentration of cells for LDH release must be determined. This is concentration of cells that gives the biggest difference in LDH levels when comparing corresponding resting and lysed samples. Serial dilutions of cells were prepared and LDH release was measured in corresponding resting and lysed samples. For each concentration of cells, the absorbance value of resting cells was subtracted from the absorbance value of lysed cells and this value was plotted against the concentration of cells. The optimal concentrations of cells for LDH release were 1 x 10^6/ml for A2780 cells and 2.5 x 10^8/ml for platelets. At these concentrations, A2780 cells and platelets show comparable LDH release upon lysis.

4.2.3 Venous and arterial shear rates cause membrane damage in A2780 cancer cells

Having established that 1 x10^6 cells/ml as the optimal concentration for monitoring LDH release by A2780 cells, the effect of venous and arterial shear rates on A2780 cells was assessed. The percentage of LDH release was calculated relative to resting and lysed A2780 cells. LDH release by A2780 cells increased in a shear rate dependent manner from 200-1500s^{-1} and in a time dependent manner from 1-10 minutes (Figure 4.4). After a 10 minute exposure period, LDH release by A2780 cells was 25 ± 3% in response to 200s^{-1} and 31 ± 4% in response to 1500s^{-1} (n = 9 - 11, Figure 4.4a). These results indicate that A2780 cells are sensitive to mechanical damage at venous and arterial shear rates. In contrast to A2780 cells, LDH release by platelets was minimal at both venous and arterial shear rates. At 200s^{-1}, % LDH release by platelets was 1 ± 1 % and 3 ± 3% at 1500s^{-1} (n = 4, Figure 4.5). LDH release

by platelets was significantly less than LDH release by A2780 at both venous and arterial shear rates ($p < 0.01$, Figure 4.5).

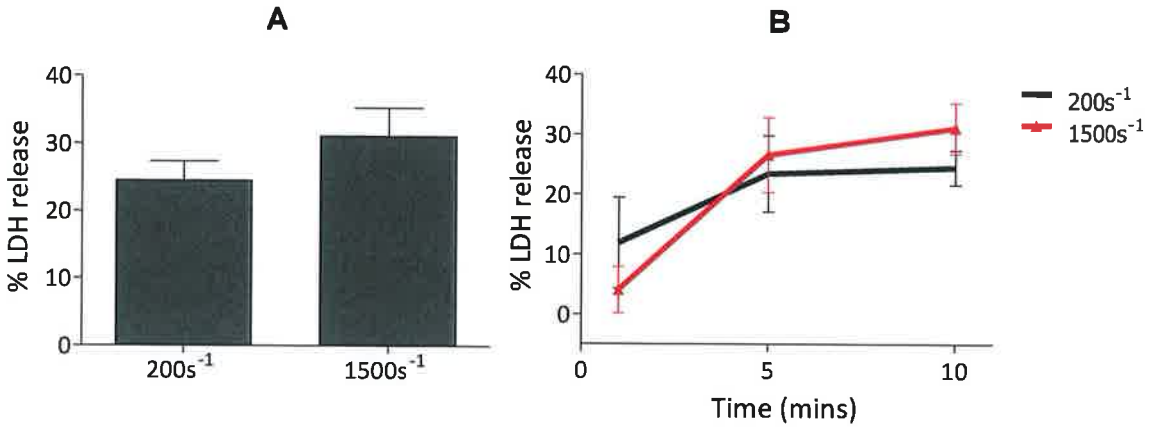

Figure 4.4: Venous and arterial shear rates cause membrane damage in A2780 cell (mean + SEM, n = 3 -11). Using a cone and plate viscometer, A2780 cells (1 x 10^6/ml) were exposed to a venous ($200s^{-1}$) and arterial ($1500s^{-1}$) shear rates for 1, 5 and 10 minutes. LDH release by A2780 cells was used as a measure of membrane damage. **(A)** After 10 minutes, LDH release by A2780 cells was significant at both 200s-1 and 1500s-1 (n = 9, mean + SEM). **(B)** LDH release by A2780 cells at $200s^{-1}$ and $1500s^{-1}$ increases in a time dependent manner, with the most significant release seen after 10 minutes.

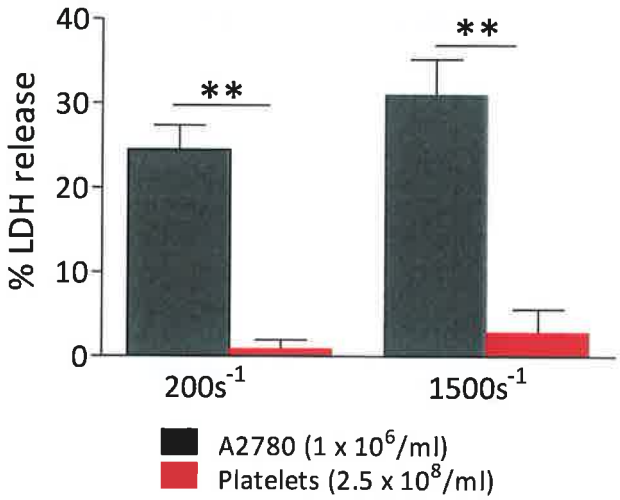

Figure 4.5: Venous and arterial shear rates do not cause membrane damage in platelets (mean + SEM, n = 4 - 9). LDH release by **A2780 cells** and platelets exposed to venous ($200s^{-1}$) and arterial ($1500s^{-1}$) shear rates for 10 minutes was compared. LDH release by platelets was minimal and significantly less than LDH release by A2780 at both venous and arterial shear rates. Data was analysed using Mann Whitney test (* = $p < 0.01$).

4.2.4 Platelets protect A2780 cells from shear induced damage at arterial shear rates

Having established that platelets adhere to A2780 cells at physiologically relevant shear rates and that A2780 cells are susceptible to shear induced membrane damage, it was next determined if platelets could protect A2780 cells from shear induced membrane damage. Since it was demonstrated that LDH release by platelets was negligible at venous and arterial shear rates, all LDH released would be attributable to A2780 cells. A2780 cells (1 x 10^6/ml) were incubated with a low (50 x 10^6/ml) or high (200 x 10^6/ml) concentration of platelets, resulting in a ratio of 1:50 or 1:200 (A2780 cell: platelets). Samples were then exposed to venous and arterial shear rates using a cone and plate viscometer.

When A2780 cells were incubated with platelets in a ratio of 1:50, there was no difference in LDH release at 200s^{-1}, compared to untreated A2780 cells (19 ± 4 % n = 6 vs. 24 ± 2% n = 11). However, when A2780 cells were incubated with platelets in a ratio of 1:200, there was a trend towards a significant decrease in LDH release by A2780 cells at 200s-1, compared to untreated cells (13 ± 5 % n = 5 vs. 24 ± 2 % n = 11, p = 0.057). In contrast to the venous shear rate of 200s^{-1}, when A2780 cells were incubated with platelets at a 1:50 or a 1:200 ratio, there was a significant decrease in LDH release at 1500s^{-1}. At 1500s^{-1}, the percentage of LDH release by A2780 cells was 6 ± 4 % at the 1:50 ratio (n = 3), 11 ± 2 % at the 1:200 ratio (n = 3), compared to 31 ± 4 % (n = 9) in untreated cells ($p \leq 0.05$). Hence, these results demonstrate for the first time that platelets can protect cancer cells from shear induced damage (Figure 4.8).

Since shear stress is a function of shear rate and viscosity, the addition of platelets to suspensions of A2780 cells could potentially increase the viscosity of the sample and therefore increase the shear stress value at 200 and 1500s^{-1}, respectively. Using the cone and plate viscometer, viscosity and shear stress measurements were obtained. There was no change in the viscosity of suspensions of A2780 cells alone or suspension of A2780 cells incubated with platelets in a ratio of 1:50 or 1:200 at

venous or arterial shear rates (Figure 4.6a & 4.7a). Correspondingly, there was also no change in the shear stress value applied to suspensions of A2780 cells alone or suspensions of A2780 cells incubated with platelets in a ratio of 1:50 or 1:200 at venous or arterial shear rates (Figure 4.6b & 4.7b). Hence, any increases or decreases in LDH release by A2780 cells in the presence of platelets could not be attributed to changes in the amount of shear stress applied.

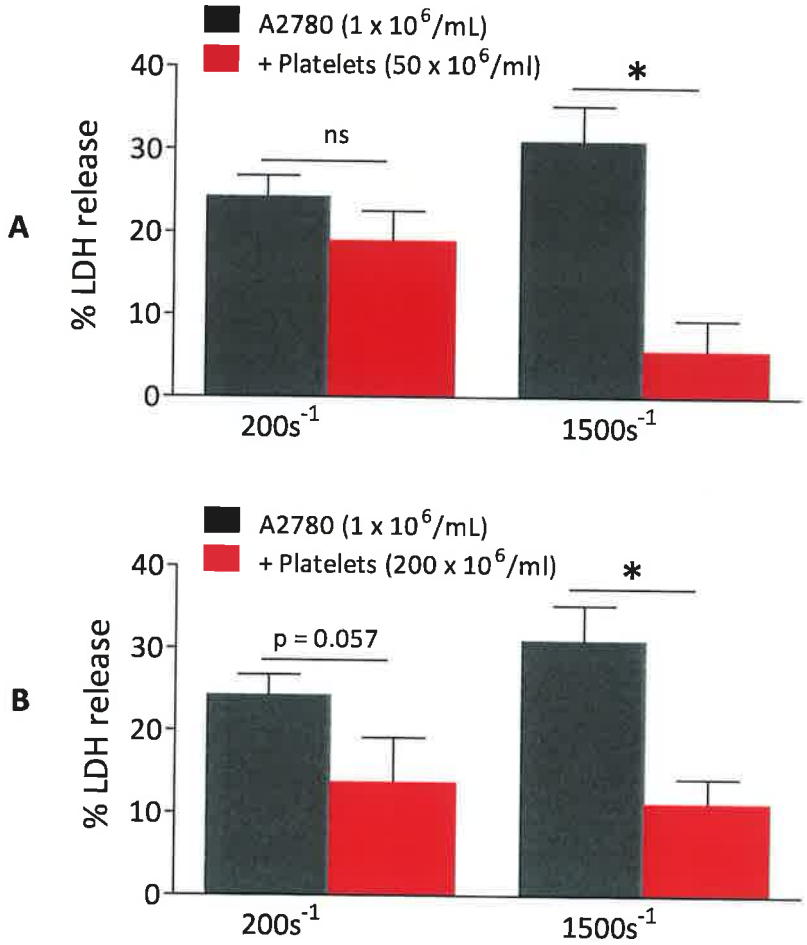

Figure 4.6: Platelets protect A2780 cells from shear induced membrane damage (mean ± SEM, n = 3 - 11). A2780 cells (1 x10^6/ml) were incubated with platelets in a ratio of 1:50 and 1:200 and subsequently exposed to 200 and 1500s^{-1} in a cone and plate viscometer for 10 minutes. **(A)** LDH release by untreated A2780 cells **(black)** and A2780 cells incubated with platelets in a ratio of 1:50 **(red)** at 200 and 1500s^{-1}. There was no difference in LDH release in untreated A2780 cells and A2780 cells incubated with platelets in a ratio of 1:50. In contrast, there was a significant decrease in LDH release by A2780 cells incubated with platelets in a ratio of 1:50 at 1500s-1 (* = $p \leq 0.05$). **(B)** LDH release by untreated A2780 cells **(black)** and in the presence of platelets in a ratio of 1:200 **(red)** at 200 and 1500s^{-1}. There was a trend towards decreased LDH release by A2780 cells in the presence of platelets at 200s^{-1} ($p = 0.057$). There was a significant decrease in LDH release by A2780 cells incubated with platelets in a ratio of 1:50 and 1:200 at 1500s-1 (* = $p \leq 0.05$). Data was analysed using a student t test.

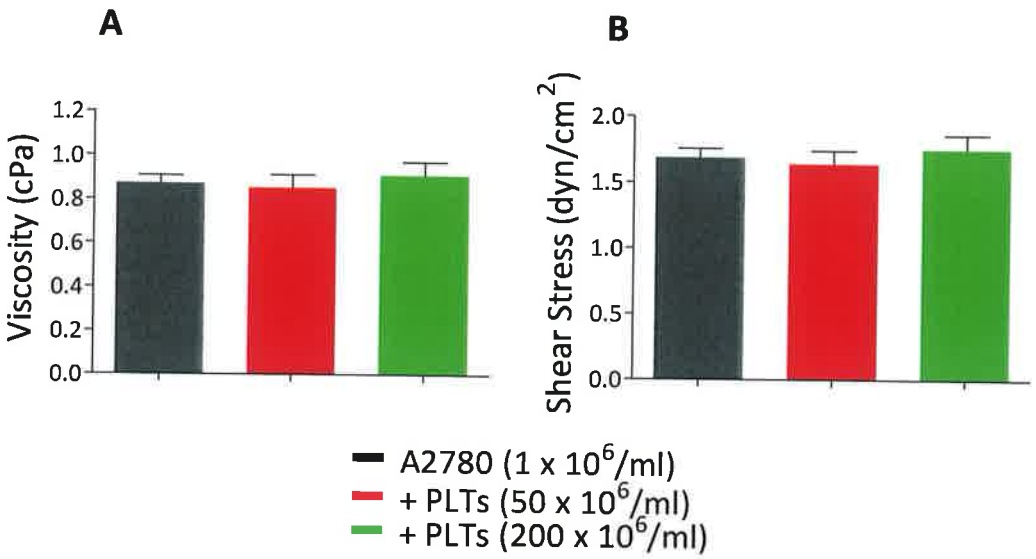

Figure 4.7: The addition of platelets to suspensions of A2780 cells does not change viscosity or shear stress measurements at venous ($200s^{-1}$) shear rates (mean + SEM, n = 4-7). In order to determine if platelets could protect cancer cells from shear induced damage at arterial shear rates, A2780 cells (1 x 10^6/ml) were exposed to $200s^{-1}$ (~ 1.5 – 2 dyn/cm^2) in a cone and platelet viscometer, in the presence of low (50 x 10^6/ml) and high (200 x 10^6/ml) concentrations of platelets. Since shear stress is a function of viscosity as well as shear rate, the addition of platelets could potentially increase the viscosity of the sample and hence the shear stress applied to A2780 cells in the experimental set up. Using the cone and plate viscometer, viscosity and shear stress measurements were obtained. **(A)** There was no significant difference in the average viscosity reading of suspensions of **A2780 cells alone**, A2780 **cells + platelets (50 x 10^6/ml)** and **A2780 cells + platelets (200 x 10^6/ml)** exposed to $200s^{-1}$ for 10 minutes. **(B)** Correspondingly, there was no difference in the shear stress value applied to **A2780 cells alone**, **A2780 cells + platelets (50 x 10^6/ml)**, or **A2780 cells + platelets (200 x 10^6/ml)** exposed to 200 s^{-1}. Data was analysed using a student t test.

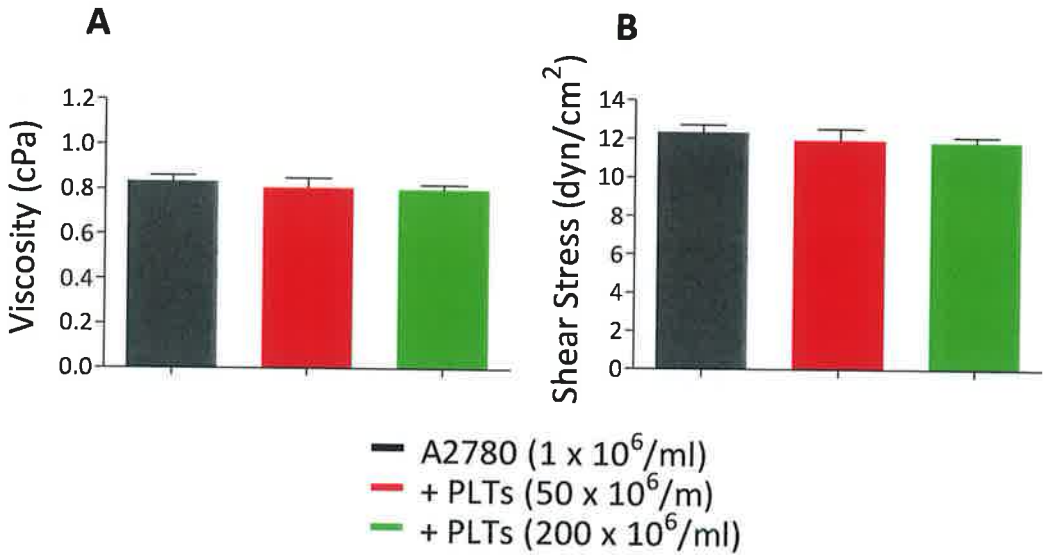

Figure 4.8: The addition of platelets to suspensions of A2780 cells does not alter viscosity or shear stress measurements at 1500s^{-1} (mean + SEM, n = 3-6). In order to determine if platelets could protect cancer cells from shear induced damage at arterial shear rates, A2780 cells (1 x 10^6/ml) were exposed to 1500s^{-1} (~ 12dyn/cm^2) in a cone and platelet viscometer in the presence of low (50 x 10^6/ml) and high (200 x 10^6/ml) concentrations of platelets. Since shear stress is a function of viscosity as well as shear rate, the addition of platelets could potentially increase the viscosity of the sample and hence the shear stress applied to A2780 cells in the experimental set up. Using the cone and plate viscometer, viscosity and shear stress measurements were obtained. **(A)** There was no significant difference in the viscosity readings of suspensions of **A2780 cells alone**, **A2780 cells + platelets (50 x 10^6/ml)** and **A2780 cells + platelets (200 x 10^6/ml)** exposed to 1500s^{-1}. **(B)** Correspondingly, there was no difference in the shear stress value applied to A2780 cells alone, **A2780 cells + platelets (50 x 10^6/ml)**, or **A2780 cells + platelets (200 x 10^6/ml)** exposed to 1500s-1. Data was analysed using a student t test.

4.2.5 A2780 cell induced platelet degranulation under venous and arterial shear conditions

These results show for the first time that platelets can protect cancer cells from shear induced damage. The short assay time (10 minutes) suggested that platelets conferred protection against shear induced damage via a fast acting mechanism, most likely linked to adherent platelets acting as a physical shield around A2780 cells, i.e. platelet cloaking. However, the protection conferred to A2780 cells by platelets was less evident at $200s^{-1}$ than at $1500s^{-1}$, even though platelet adhesion to A2780 cells was comparable at both shear rates (Figure 4.2). It was hypothesised that A2780 cells were inducing increased platelet granule release under arterial shear conditions. Platelets are rich sources of growth factors, angiogenic factors, and adhesive proteins that could influence cell viability under conditions of stress. To assess this, A2780 cells were exposed to venous and arterial shear rates in a cone and plate viscometer in the presence of platelets in the previously described ratios of 1:50 and 1:200. Platelet degranulation was assayed by P-selectin expression.

At a ratio of 1:50, there was no difference in A2780 cell induced platelet P-selectin expression at 0, 200, and $1500s^{-1}$ (Figure 4.9a). At a ratio of 1:200, A2780 cells induced significantly more P-selectin expression at 200 than at $0s^{-1}$, (Figure 4.9b). However, there was no significant difference in A2780 induced platelet degranulation at 200 and $1500s^{-1}$ at any ratio. For example, in a ratio of 1:200, A2780 cell induced platelet P-selectin expression was 18 ± 4 % at $200s^{-1}$ and 18 ± 2 % at $1500s^{-1}$, respectively (Figure 4.9b). This suggests that the protection conferred by platelets against shear induced damage in A2780 cells is not attributable to increased or decreased platelet granule release. A2780 cell induced platelet aggregation was also assayed. There was a trend toward increased A2780 cell induced platelet aggregation at $200s^{-1}$ compared to $1500s^{-1}$ (Figure 4.10 a & b). In a ratio of 1:200, A2780 cell induced platelet aggregation was 30 ± 8 % at $200s^{-1}$ compared to 10 ± 2 % at $1500s^{-1}$ (n = 3). This difference in platelet aggregation is likely caused by the propensity of weakly bound platelet aggregates to disaggregate as higher shear rates [212].

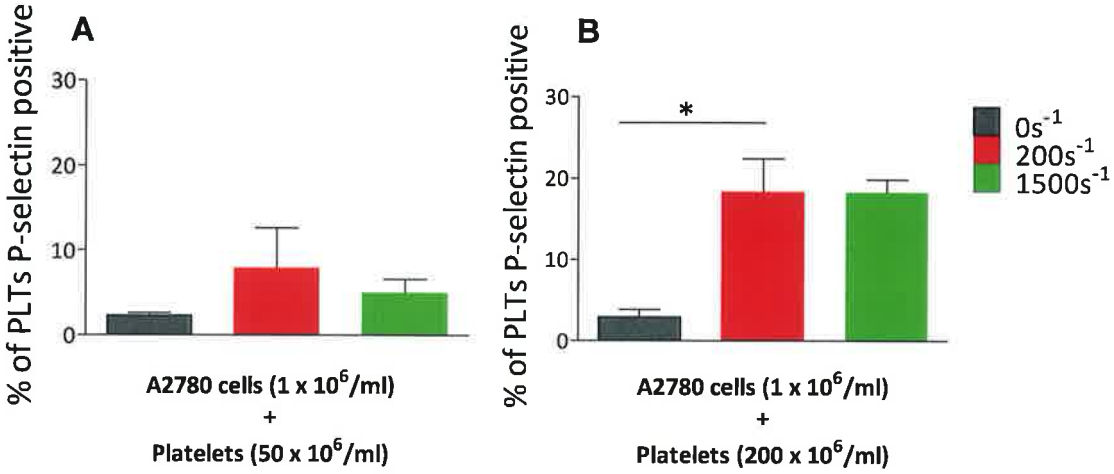

Figure 4.9: A2780 cell induce comparable levels of platelet degranulation (P-selectin expression) at venous and arterial shear rates (mean + SEM, n = 3). A2780 cell (1 x 10^6/ml) induced platelet activation and degranulation at **0**, **200**, and **1500s^{-1}** was assayed based on platelet P-selectin expression. (A) In a low concentration suspension of platelets (50 x 10^6/ml) A2780 cells induce minimal P-selectin expression at 0, 200, and 1500s^{-1}. (B) In a high concentration suspension of platelets (200 x 10^6/ml) A2780 cells induce significantly more P-selectin expression at 200s^{-1} compared to 0s^{-1} (* = $p \leq 0.05$). However, there was no difference in A2780 cell induced platelet P-selectin expression at 200 and 1500s-1. Data was analysed using a paired t test.

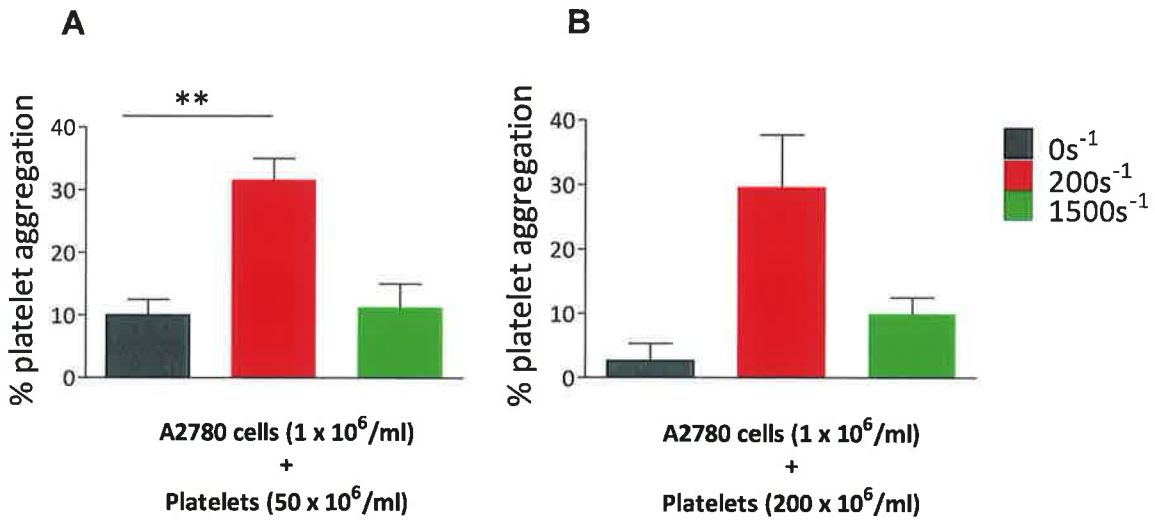

Figure 4.10: A2780 cell induce significantly more platelet aggregation at venous shear rates (mean + SEM, n = 3). A2780 cell (1 x 10^6/ml) induced platelet aggregation at **0**, **200**, and **1500s^{-1}** was assayed based on the decrease in platelet count following platelet aggregation. (A) In a low concentration of platelets (50 x 10^6/ml), A2780 cell induce significantly more platelet aggregation at **200s^{-1}** than **0s^{-1}**. There was no significant difference between platelet aggregation at 0 and 1500s^{-1}. (B) In a high concentration of platelets (200 x 10^6/ml), there was no significant difference in A2780 induced platelet aggregation at 0, 200, or 1500s^{-1}. Data was analysed using a paired t test.

4.3 Discussion

It is frequently suggested that platelets can protect circulating cancer cells form shear induced damage. However, there is no evidence to support or oppose this hypothesis. It was hypothesised that through the phenomenon of platelet cloaking (platelet tumour cell adhesion), platelets would act as a physical shield around tumour cells, and hence protect against shear forces. To assess this, an *in vitro* model of shear induced damage in A2780 ovarian cancer cells was established. Using this model, it was demonstrated for the first time that platelets are capable of protecting cancer cells under certain physiologically relevant conditions.

First, platelets were shown to cloak or adhere to A2780 cells. Platelet adhesion to A2780 cancer cells under venous and arterial shear conditions was assessed (Figure 4.2). In chapter 3, platelets were shown to adhere to A2780 cells under static conditions (Figure 3.1). Other investigators have demonstrated platelet adhesion to various cancer cell lines under static conditions [28, 30, 213]. However, shear stress conditions generated by blood flow are known to critically affect the adhesive interactions of cancer cells. For example, while melanoma cells adhere to immobilised collagen type I under static conditions, they fail to adhere under flow conditions [214]. McCarthy *et al* have also shown that colon cancer cell adhesion to immobilised platelets decreases as shear stress increases, with minimal colon cancer cell adhesion seen after the shear stress increased to 1.4 dyn/cm^2 [29]. Platelets were clearly shown to adhere to A2780 cells over a range of shear rates (0 - 1500s^{-1}) with platelet adhesion increasing in a platelet count dependent manner.

Second, it was demonstrated that A2780 cells are susceptible to shear induced damage. Venous and arterial shear rates caused an increase in LDH release by A2780, indicative of shear induced membrane damage. LDH release increased in a shear rate dependent manner from 200-1500s^{-1} and in a time dependent manner from 1-10 minutes (Figure 4.4). Along with the work of Brooks[210] showing shear induced damage of cancer cells *in vitro* and Wyckoff *et al* showing shear induced

membrane fragmentation of cancer cells *in vivo*[211], these results suggest that shear stress could be an important rate regulating factor in metastasis. Shear stress could also regulate metastasis in other ways. In order for circulating cancer cells to leave the circulation and form metastatic foci, they must attach to the endothelium of blood vessels and extravasate into the extracellular space. As discussed previously, shear stress adversely affects the adhesive properties of cancer cells. Giavazzi *et al* studied cancer cell adhesion to endothelial cells under flow conditions. Colon, breast, and ovarian cancer cell lines failed to adhere to endothelial cells at any shear stress levels (0.3 – 3 dyn/cm^2), while melanoma and osteosarcoma cell lines only adhered at low shear stress levels[215].

These results are consistent with the idea of shear stress as an important regulator of pathobiology, most notably in the progression of atherosclerosis. Atherosclerosis is a complex chronic inflammatory condition affecting the arterial circulation. It results in the development of atherosclerotic plaques, asymmetrical thickenings of the intima of muscular and elastic arteries, consisting of extracellular matrix proteins, lipids, monocyte derived macrophages, T-lymphocytes, and smooth muscle cells [159, 160]. Atherosclerotic plaques co-localise with regions of low shear stress (~ 4 dyn/cm^2), such as the carotid bifurcation, while regions of higher shear stress (~12 dyn/cm^2) are protected [204, 205]. Biomechanical signalling caused by elevated shear stress levels confers an atheroprotective phenotype in endothelial cells. Endothelial cells exposed to high shear stress are characterised by decreased expression of vasoconstrictors, growth factors, inflammatory mediators, oxidants, and adhesion molecules, and the increased expression of vasodilators, antioxidants, and antiplatelet agents [204, 205].

After demonstrating shear induced damage in A2780 cells, the effect of venous and arterial shear rates on membrane damage in platelets was assessed. In contrast to A2780 cells and as expected, venous and arterial shear rates caused minimal LDH release by platelets even after prolonged exposure to a constant shear rate (Figure 4.5) Blood cells such as platelets and erythrocytes are characterised by specialised cytoskeletal networks linked to plasma membrane protein that regulate membrane

integrity in the face of shear stress conditions. For example, in platelets, disruption of the interaction between membrane GPIbα and cytoskeletal filamin causes the loss of membrane integrity under pathological shear conditions (5000 - 40000s-1), leading to membrane fragmentation and cell disintegration [216].

Third, having established that platelets cloak A2780 cells under physiologically relevant shear conditions and that A2780 cells are susceptible to shear induced membrane damage, it was next determined if platelet cloaking (platelet adhesion) could protect A2780 cells from shear induced damage. Again, membrane damage was assessed by LDH release. Since it had been shown that LDH release by platelets was minimal at both venous and arterial shear rates, all LDH could be attributed to A2780 cells in the system. Platelet cloaking caused a significant decrease in LDH release by A2780 cells at an arterial shear rate ($p \leq 0.05$) and there was a trend towards decreased LDH release by A2780 cells at a venous shear rate ($p = 0.057$), demonstrating for the first time that platelet cloaking can confer protection against shear induced damage in cancer cells under physiologically relevant conditions (Figure 4.8).

The short assay time (10 minutes) suggested that platelets confer protection to A2780 cells against shear induced damage through a fast acting mechanism, likely linked to adherent platelets acting as a physical shield around A2780 cells. However, the protection conferred to A2780 cells by platelets was less evident at $200s^{-1}$ than at $1500s^{-1}$, even though platelet adhesion to A2780 cells was comparable at both shear rates. Platelet releasate is rich in growth, angiogenic, and adhesion factors. Work from our lab has previously shown that platelet releasate modulates the invasive behaviour of OC-1 oesophageal cancer cells under shear conditions. Following pretreatment with platelet releasate (30 minutes), OC-1 cells showed significantly increased adhesion to endothelial cells under dynamic flow conditions. This effect was dependent on the interaction of platelet released vitronectin and fibronectin, and their receptor integrin αvβ3 on OC-1 cells [217].

Based on the ability of platelet secreted proteins to modulate tumour cell behaviour on a relatively short time scale, it was hypothesised that the increased protection of A2780 cells at arterial shear rates could be attributable to increased platelet activation and granule release at $1500s^{-1}$. However, it was found that there was no difference in platelet granule release at $200s^{-1}$ and $1500s^{-1}$, as measured by platelet P-selectin expression (Figure 4.9). This suggests that the protection conferred to A2780 under shear conditions is not related to increased granule release. Interestingly, recent work has demonstrated that the platelet granule protein TGFβ can undergo shear induced activation, and activation increases with an increasing shear rate[218]. Platelets are a rich source of TGFβ, containing 40 to 100 times more than other cells in their alpha granules[219]. Platelet derived TGFβ has been shown to be extremely important in facilitating metastasis. Labelle *et al* have demonstrated that the genetic depletion of platelet derived TGFβ1 conferred protection against metastasis in an animal model[90]. Whether shear induced activation of platelet secreted proteins such as TGFβ could contribute to protection against shear induced damage remains to seen.

In conclusion, this study demonstrates for the first time that platelets can protect cancer cells from mechanical damage resulting from shear stress. The results show that the protective effect of platelets is most evident at arterial shear rates compared to venous shear rates.

Chapter 5: Platelet hyperreactivity in metastatic cancer patients

5.1 Introduction

Thrombosis is a frequent complication of cancer. It is the second leading cause of death in cancer patients after metastasis[132]. The link between cancer and thrombosis has been known for nearly two centuries. Clinically detectable thrombosis is seen in 15 % of cancer patients [136-138]. Up to 50% of patients who have died of non thrombotic complications also show subclinical evidence of thrombosis upon autopsy [133, 139]. Cancer patients are at increased risk of recurrent thrombosis compared to non-cancer patients, even when on oral anticoagulant therapy [140, 141]. Patients with idiopathic thrombosis (thrombosis of unknown cause) are considered at high risk for developing or being subsequently diagnosed with cancer [135, 142]. DVT of the leg is the most common clinical manifestation of thrombosis in cancer patients. However, DVT of the arm, pulmonary embolism, cerebral sinus thrombosis, disseminated intravascular coagulation, non bacterial thrombotic endocarditis, and arterial thrombosis have all been documented in cancer patients, but to a lesser extent [143-147].

In patients with cancer, tumour site, tumour stage, therapy (chemotherapy, radiotherapy, hormonal therapy), and surgery are the major determinants of thrombosis. However, modulation of the haemostatic system during cancer progression is believed to make a significant contribution to thrombosis in this cohort [148]. It is well established that patients with cancer show increased coagulation activation, as evidenced by increased levels of fibrinogen breakdown products and coagulation proteins [164-167]. In contrast, there is a paucity of studies assessing whether platelet function is altered in cancer patients. In response to physiological agonists, platelets become activated (e.g. release of granule content, integrin αIIbβ3 becomes activated), then aggregate together. These events can be measured *ex vivo* using a number of platelet function assays. Based on platelet function testing, the

concept of platelet hyperreactivity has been recognised and can be defined as an exaggerated platelet response (e.g.) increased platelet activation or aggregation in response to low doses of platelet agonists [168]. Rather than being a laboratory artefact, measures of increased platelet reactivity, increased αIIbβ3 activation[169], increased spontaneous platelet aggregation [170], and increased ADP and epinephrine induced platelet aggregation [171, 172], have been associated with an increased incidence of major adverse cardiovascular events.

In line with abnormalities in secondary haemostasis, it was hypothesised that patients with cancer would display increased platelet reactivity, a factor that could contribute to the increased incidence of thrombosis in this cohort. To assess this hypothesis, blood samples were collected from 13 patients with advanced metastatic cancer and 10 healthy controls and platelet function testing was performed. Patients with metastatic cancer were chosen since abnormalities in coagulation are known to increase with disease progression [167]. Hence, patients with advanced disease (of any origin) would likely have more overt abnormalities in platelet reactivity compared to patients with localised disease. In this study, it is demonstrated that patients with metastatic cancer display significantly increased platelet reactivity, as evidenced by increased agonist induced platelet aggregation, increased spontaneous platelet aggregation, and increased agonist induced platelet activation (P-selectin expression).

5.2: Results

5.2.1 Increased agonist induced platelet aggregation in metastatic cancer patients

In collaboration with Dr Bryan Henessey, blood samples were obtained from 13 patients with advanced untreatable metastatic cancer, who were receiving palliative care as in-patients in the Oncology department at Beaumont Hospital. Since disease progression had advanced beyond treatment in nearly all patients, only one patient was undergoing chemotherapy or radiotherapy at the time of platelet function testing. The patient cohort was extremely heterogeneous in cancer type and medication use. Patient demographic information is detailed in Table 5.1. Blood samples from healthy controls were obtained from volunteers at the Royal College of Surgeons in Ireland or Beaumont Hospital.

To determine if platelet reactivity is increased in metastatic cancer patients, agonist induced platelet aggregation, spontaneous platelet aggregation, and agonist induced platelet activation was assayed. Platelet aggregation responses in patients with metastatic cancer patients (n = 13) and healthy controls (n = 10) were measured using a 96 well plate modification of classical light transmission aggregometry. The assay tests aggregation responses to multiple concentrations of five platelet agonists and hence, tests multiple platelet activation pathways simultaneously. The platelet agonists used are arachidonic acid *(acts on the TxA2 receptor)*, collagen *(GPVI and α2β1)*, ADP *(P2Y1 and P2Y12 receptors)*, epinephrine *(adrenergic α21 receptor)*, and PAR-1 activating peptide *(PAR-1 receptor)*.

For each agonist, there was a dose dependent increase in platelet aggregation for both cohorts (Figure 5.1 - 5.5). Compared to healthy controls, metastatic cancer patients displayed global platelet hyperreactivity. Platelet aggregation was significantly increased in response to all agonists (arachidonic acid, collagen, ADP, epinephrine, and PAR-1 activating peptide) (Figure 5.1 - 5.5). In response to arachidonic acid, metastatic cancer patients displayed significantly increased platelet

aggregation in response to 5.5, 11.8, and 23.6 ug/ml arachidonic acid. For example, in response to 23.6 µg/ml arachidonic acid, % platelet aggregation was 10 ± 2 % in metastatic cancer patients (n = 13), compared to 3 ± 1% in healthy controls (n = 10, $p \leq 0.05$, Figure 5.1). Collagen induced aggregation responses were increased in metastatic cancer patients in response to 2.2, 4.4, 8.9, 17.8, 35.6, and 71.25 µg/ml collagen (Figure 5.2). For example, in response to 17.8 µg/ml of collagen, % platelet aggregation was 66 ± 4 % in metastatic cancer patients (n = 13) compared to 7 ± 2 % in healthy controls (n =10, $p \leq 0.05$). In response to 72.5 µg/ml of collagen, % platelet aggregation was 83 ± 2 % in metastatic cancer patients (n = 13) compared to 66 ± 4 % in healthy controls (n = 10, $p \leq 0.05$). ADP induced platelet aggregation was increased in metastatic cancer patients in response to 0.15, 0.3, 0.6, 1.25, and 2.5 µM ADP (Figure 5.3). For example, in response to 1.25 µM ADP, % platelet aggregation was 56 ± 5 % in metastatic cancer patients (n = 13) compared to 32 ± 6 % in healthy controls (n = 10, $p \leq 0.05$). Epinephrine induced platelet aggregation was increased in metastatic cancer patients in response to 1.25, 5, and 20 µM epinephrine (Figure 5.4). For example, in response to 5 µM epinephrine, % platelet aggregation was 71 ± 4 % in metastatic cancer patients (n = 13) compared to 46 ± 5 % in healthy controls (n = 10, $p \leq 0.05$). PAR-1 activating peptide induced platelet aggregation was increased in response to 0.15, 0.3 and 20 µM PAR-1 activating peptide (Figure 5.5). For example, in response to 20 µM PAR-1 activating peptide, % platelet aggregation was 88 ± 2 % in metastatic cancer patients (n = 13) compared to 80 ± 1 % in healthy controls (n = 10).

5.2.2 Increased spontaneous platelet aggregation in metastatic cancer patients

Spontaneous platelet aggregation in metastatic cancer patients and healthy controls was also measured using the 96 well plate modification of light transmission aggregometry. This is aggregation that occurs in the platelet rich plasma control wells over the assay time course in the absence of agonist stimulation, but in the presence of mechanical stimulation (i.e.) shaking (Figure 5.6). In line with increased agonist induced platelet aggregation, spontaneous platelet aggregation was also

significantly increased in metastatic cancer patients (8 ± 1 %, n = 13) compared to healthy controls (1 ± 1 %, n = 10, $p \leq 0.05$).

Table 5.1 Patient demographic information.

Age (years)	64 ± 4
Male (%)	60% (8/13)
Platelets (x 10^6/ml)	328 ± 34
White blood cell (x 10^6/ml)	8 ± 1
C reactive protein (mg/l)*	27 ± 7
Cancer type (all metastatic)	ovarian (x 2), pancreatic (x 2), breast (x 2), lung, colorectal, melanoma, chrondosarcoma, gastric, oesophageal, glioblastoma multiforme
Medications	
Chemotherapy **	7.5 % (1/13)
Aspirin	15%
Anticoagulants	37.5%
Paracetamol	30%
Calcium channel blocker	22.5%
Beta blockers	22.5%
Statins	15%
Angiotensin inhibitor***	15%
Proton pump inhibitor	60%
Nitrates	7.5%
Loop diuretic	15%
Glucocorticosteroids	30%
Opiates	60%
Selective serotonin reuptake inhibitors	22.5%
Antibiotics	30%

Data is presented as mean ± SEM or the % of patients. Demographic information was not obtained from healthy volunteers in line with the RCSI and Beaumont hospitals ethics policy. * C-reactive protein levels were only available for 10 patients. ** Patient receiving paclitaxel and carboplatin. *** Angiotensin II receptor blockers or Angiotensin converting enzyme inhibitor.

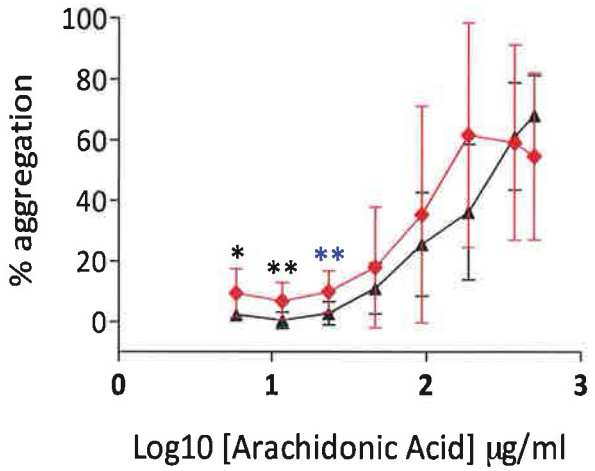

Figure 5.1: Increased arachidonic acid induced platelet aggregation in metastatic cancer patients (mean ± standard deviation). Platelet aggregation responses to multiple concentrations of arachidonic acid were measured in **metastatic cancer patients (n = 13)** and compared to **normal healthy controls (n = 10).** Arachidonic acid is converted via prostaglandin synthesis to thromboxane A2, which induces platelet activation and aggregation via the TxA2 (TP) receptor. % platelet aggregation is increased in metastatic cancer patients in response to 5.5, 11.8, and 23.6 µg/ml arachidonic acid (*= $p \leq 0.05$, student t test; * = $p \leq 0.05$, Mann Whitney test)

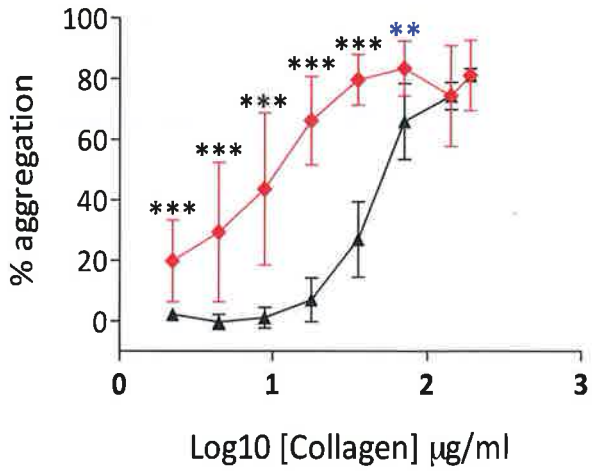

Figure 5.2: Increased collagen induced platelet aggregation in metastatic cancer patients (mean ± standard deviation). Platelet aggregation responses to multiple concentrations of collagen were measured in **metastatic cancer patients (n = 13)** and compared to **normal healthy controls (n = 10).** Collagen induces platelet activation and aggregation via GPVI and α2β1. % platelet aggregation was significantly increased in metastatic cancer patients in response to multiple concentrations of collagen. (*= $p \leq 0.05$, student t test; * = $p \leq 0.05$, Mann Whitney test)

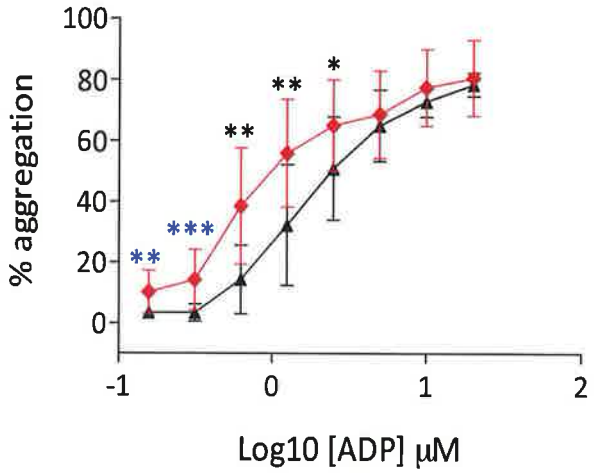

Figure 5.3: Increased ADP induced platelet aggregation in metastatic cancer patients (mean ± standard deviation). Platelet aggregation responses to multiple concentrations of ADP were measured in **metastatic cancer patients (n = 13)** and compared to **normal healthy controls (n = 10).** ADP induces platelet aggregation via the P2Y1 and P2Y12 receptors. % platelet aggregation was significantly increased in metastatic cancer patients in response to 0.15, 0.3, 0.6, 1.25, and 2.5 µM ADP (*= p ≤ 0.05, student t test; * = p ≤ 0.05, Mann Whitney test).

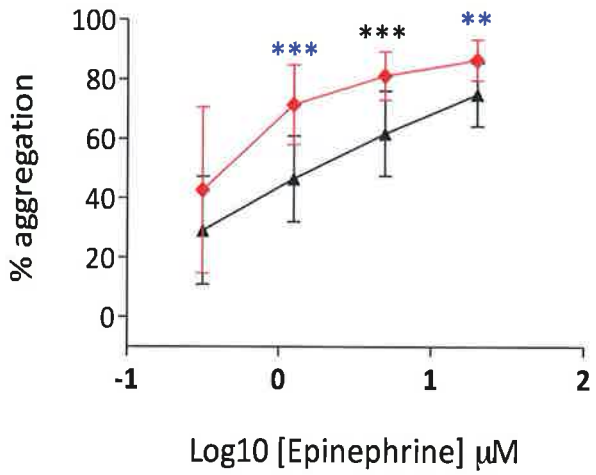

Figure 5.4: Increased epinephrine induced platelet aggregation in metastatic cancer patients (mean ± standard deviation). Platelet aggregation responses to multiple concentrations of epinephrine were measured in **metastatic cancer patients (n = 13)** and compared to **normal healthy controls (n = 10).** Epinephrine induces platelet activation and aggregation via the α2 adrenergic receptor. % platelet aggregation was significantly increased in metastatic cancer patients in response to 1.25, 5, and 20 µM epinephrine (*= p ≤ 0.05, student t test; * = p ≤ 0.05, Mann Whitney test).

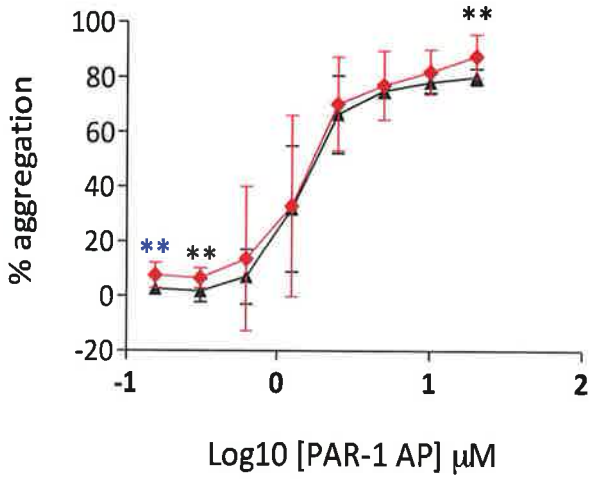

Figure 5.5: Increased PAR-1 activating peptide induced platelet aggregation in metastatic cancer patients (mean ± standard deviation). Platelet aggregation responses to multiple concentrations of PAR-1 activating peptide were measured in **metastatic cancer patients (n = 13)** and compared to **healthy controls (n = 10)**. PAR-1 activating peptide induced platelet aggregation and activation via the thrombin receptor PAR-1. % platelet aggregation was significantly increased in metastatic cancer patients in response to 0.15, 0.3, and 20 μM PAR-1 activating peptide (*= $p \leq 0.05$, student t test; * = $p \leq 0.05$, Mann Whitney test).

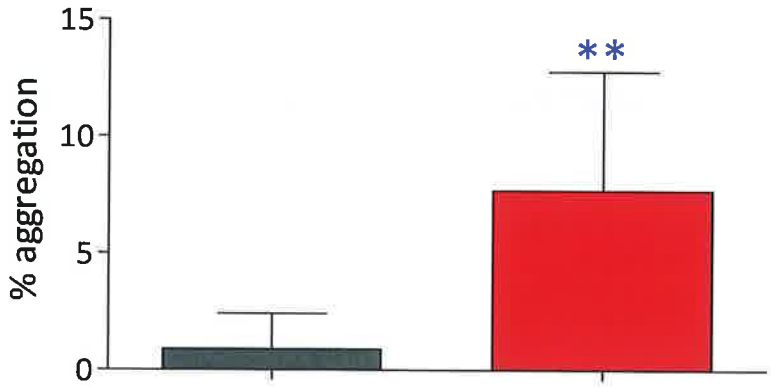

Figure 5.6: Spontaneous platelet aggregation is significantly increased in metastatic cancer patients (mean ± standard deviation). Spontaneous platelet aggregation responses were measured in **metastatic cancer patients (n = 13)** and compared to **healthy controls (n = 10).** Spontaneous platelet aggregation is platelet aggregation occurring in the absence of agonist stimulation, but in the presence of mechanical stimulation (i.e.) shaking. Spontaneous platelet aggregation was increased in metastatic cancer patients (*= $p \leq 0.05$, Mann Whitney test).

5.2.3: Significantly increased agonist Induced platelet activation in metastatic cancer patients

Agonist induced platelet activation in whole blood samples was measured by flow cytometry based on P-selectin expression. P-selectin is stored internally on alpha granules in resting platelets and translocated to the surface upon platelet activation. It is then easily detectable by flow cytometry using a labelled anti human CD62P (P-selectin) antibody. P-selectin expression induced by three different platelet agonists was assayed. The agonists used were ADP (1 and 5 μM), ADP & epinephrine in combination (1 and 5 μM), and PAR-1 activating peptide (1 and 2 μM). The concentrations of agonists used were chosen based on preliminary experiments designed to identify concentrations of agonists that induced low (10 - 30 %) and intermediate (30 - 50%) levels of P-selectin expression in healthy controls.

As well as increased agonist induced platelet aggregation and increased spontaneous platelet aggregation, metastatic cancer patients also displayed significantly increased P-selectin expression in response to agonist stimulation compared to healthy controls (Figure 5.7). In response to 5 μM ADP, the % of platelets P-selectin positive was 56 ± 5 % in metastatic cancer patients (n = 13) compared to 30 ± 5 % in healthy controls (n = 10, $p \leq 0.05$). In response to 1 μM ADP and epinephrine in combination, the % of platelets P-selectin positive was 51 ± 5 % in metastatic cancer patients (n = 13) compared to 22 ± 4 % in healthy controls (n = 10, $p \leq 0.05$). In response to 2 μM PAR-1 activating peptide, the % of platelets P-selectin positive was 59 ± 8 % in metastatic cancer patients (n = 13) compared to 28 ± 6 % in healthy controls (n = 10, $p \leq 0.05$). There was no significant difference in the % of platelets P-selectin positive in resting samples from metastatic cancer patients (1 ± 0.2 %, n = 13) and healthy controls (0.3 ± 0.1 %, n = 10). There was also no significant difference in the % of platelets P-selectin positive in response to 20 μM PAR-1 activating peptide between metastatic cancer patients (87 ± 2 %, n = 13) and healthy controls (89 ± 2 %, n = 10). This concentration of PAR-1 activating peptide is known to induce maximal platelet P-selectin expression and was included as a positive control for platelet P-selectin expression.

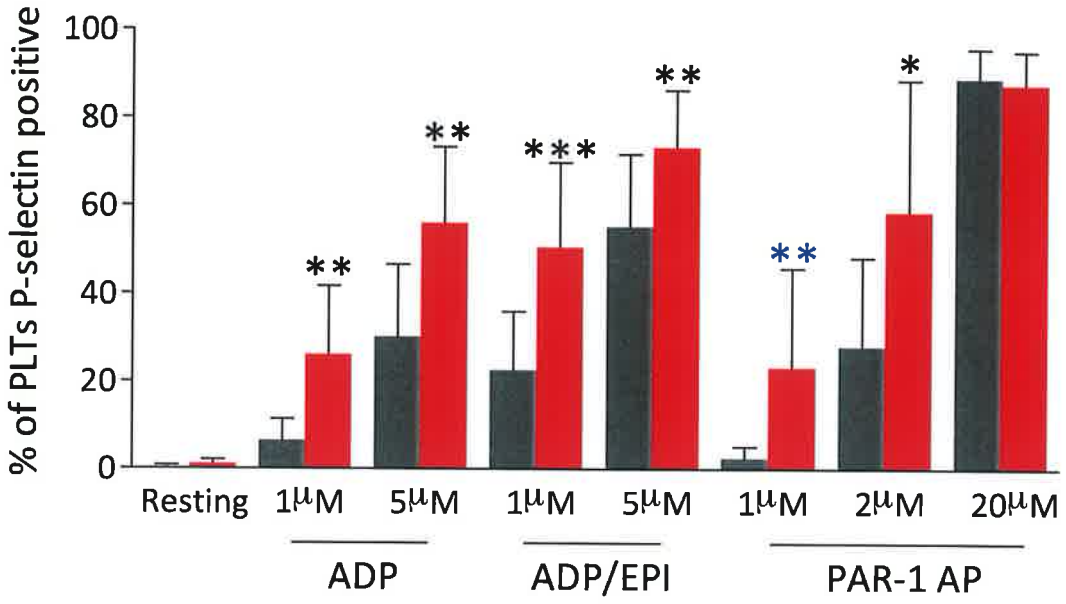

Figure 5.7: Agonist induced P-selectin expression is significantly increased in metastatic cancer patients (mean + standard deviation). Using flow cytometry and a labelled anti P-selectin antibody, agonist induced P-selectin expression were measured in **metastatic cancer patients (n = 13)** and compared to **normal healthy controls (n = 10).** Results are expressed as the % of platelet P-selectin positive relative to an isotype control. Agonist induced P-selectin expression was increased in metastatic cancer patients in response to 1 and 5 μM ADP, 1 and 5 μM ADP and epinephrine in combination, and 1 and 2 μM PAR-1 activating peptide (*= $p \leq 0.05$, student t test; * = $p \leq 0.05$, Mann Whitney test).

5.3 Discussion

In this study, it is demonstrated that patients with metastatic cancer display significantly increased spontaneous platelet aggregation, agonist induced platelet aggregation, and agonist induced platelet activation, suggestive of platelet hyperreactivity (Figure 5.1 - 5.7). In metastatic cancer patients, platelet hyperreactivity is global, seen in response to multiple platelet agonists across different assays of platelet function.

Spontaneous platelet aggregation was increased in metastatic cancer patients (Figure 5.6). This is platelet aggregation in response to mechanical stimulation, but in the absence of soluble agonist stimulation. Increased spontaneous platelet aggregation is indicative of increased platelet reactivity and has been shown to be associated with an increased incidence of thrombosis and poorer prognosis. Trip *et al* studied 149 patients with recent myocardial infarction. The patients were stratified into 3 groups based on spontaneous platelet aggregation responses. The incidences of the end point (cardiac death or nonfatal myocardial infarction) over 5 years were compared. The 3 groups were classified as positive (SPA within 10 minutes), intermediate (SPA within 10- 20 minutes), or negative (no SPA). The end point occurred in 6.4% of the negative group, 10.3% of the intermediate group, and remarkably, 34.6 % of the positive SPA group [170].

Metastatic cancer patients also displayed increased platelet activation and aggregation compared to healthy controls. Using a 96 well plate modification of light transmission aggregometry, increased platelet aggregation in response to arachidonic acid (Figure 5.1), collagen (Figure 5.2), ADP (Figure 5.3), epinephrine (Figure 5.4), and PAR-1 activating peptide (Figure 5.5) was demonstrated. This assay was developed and optimised in RCSI, and platelet function has been extensively characterised in normal healthy controls. Previously, the assay has been used to study platelet function in different disease cohorts, and increased platelet reactivity

has been demonstrated in HIV, rheumatoid arthritis, and cardiovascular disease patients, all patient cohorts at increased risk of thrombosis [220-225].

Using an assay of P-selectin expression, increased agonist induced platelet activation in metastatic cancer patients was also observed (Figure 5.7). Using a similar assay of P-selectin expression, Furman *et al* showed increased agonist induced platelet activation in coronary artery disease patients, another cohort of patients at increased risk of thrombosis [226]. Increased agonist induced platelet activation has also been associated with increased thrombosis. Kabbani *et al* studied 112 patients undergoing percutaneous coronary intervention [169]. Patients were classified into high or low responders (above or below the median response) based on the extent of platelet integrin αIIbβ3 activation in response to 0.2 μM ADP. The incidence of the composite end point (myocardial infarction, urgent revascularisation, or repeat revascularization) was compared in the two groups at 90 day follow up. The end point occurred in 7.1 % of low responders, but in 26.8 % of the high responders ($p = 0.01$).

While studies suggest a link between increased platelet reactivity and an increased incidence arterial thrombosis, arterial thrombosis in the absence of atherosclerotic or iatrogenic causes is quite uncommon in cancer patients[145]. Rather, venous thrombosis is the most prevalent thrombotic complication in malignancy [133, 134, 138, 227, 228]. Historically, venous thrombosis and arterial thrombosis have been considered distinct pathological events. Venous thrombosis under low shear conditions is traditionally associated with red cell and fibrin rich thrombi, whereas arterial thrombosis under high shear conditions are associated with platelet rich thrombi [229, 230]. This is reflected in the use of antiplatelet agents to treat arterial thrombosis and the use of anticoagulants to treat venous thrombosis [220, 221].

However, experimental models of venous thrombosis, clinical data and analysis of trials of antiplatelet agents suggest a role for platelets in the pathology of venous thrombosis. In a rabbit model, *in vivo* imaging of venous thrombosis shows the accumulation of platelets at the head of the thrombus which is later followed by the

accumulation of red blood cells leading to the characteristic 'red' clot associated with venous thrombi [231]. In a rat model, Herbert *et al* induced venous thrombosis by ligation of the vena cava in rats administered tissue factor (to increase blood hypercoagulability). In this model, thrombocytopenia was associated with reduced venous thrombus formation in rats treated with a low dose of tissue thromboplastin[232]. More recently, von Bruhl *et al* have developed a model of stasis induced venous thrombosis similar to the pathology of DVT in humans [233]. In the model, the induction of stasis causes an inflammatory endothelial phenotype that initiates the accumulation of leukocytes that initiate fibrin formation. Platelets are critical for the propagation of DVT, as they support the accumulation of leukocytes and the formation of neutrophil extracellular traps (webs of DNA, histones, and neutrophils granule content), which trigger FXII dependent thrombus formation [233]. In the setting of cancer, Ay *et al* have shown an association between increased soluble P-selectin levels (a marker of platelet activation) in cancer patients and an increased incidence of venous thrombosis [148]. Clinical trials of the antiplatelet agent aspirin have demonstrated its benefit in the prevention of primary and recurrent venous thrombosis [234-236].

Interestingly given these results, increased platelet reactivity has also been associated with an increased incidence of venous thrombosis. Large platelets are known to be significantly more reactive than smaller platelets, characterised by increased *ex vivo* platelet aggregation, shortened bleeding time, and higher levels of platelet surface receptors [237-239]. In a Norwegian based population study, Braekkan *et al* found that increased platelet size (measured as mean platelet volume) is a risk factor for venous thrombosis [240]. Hence, platelet hyperreactivity seen in this cohort of metastatic cancer patients could contribute to an increased incidence of venous thrombosis.

The underestimation of the role of platelets in venous thrombosis is also reflected in the paucity of studies of platelet function in cancer patients. A few studies have shown increased levels of markers of *in vivo* platelet activation (e.g.) platelet factor 4, beta thromboglobulin, and thromboxane A2 in serum samples from lung, breast

cancer, and head and neck cancer patients[241-244]. ADP induced aggregation is increased in nasopharyngeal and breast cancer [245, 246]. Mantur *et al.* reported slightly increased P-selectin response to ADP and PAR-1 activating peptide in renal cancer patients compared to healthy controls[247]. Thus, this study is one of the first to extensively characterise platelet function in patients with metastatic cancer. The results of the study are strengthened by the use of different platelet function assays and multiple platelet agonists. Platelet hyperreactivity in this cohort of metastatic cancer patients is global rather than unique to a single platelet signalling pathway. It is seen in response to multiple agonists across different platelet function assays.

The cohort of patients studied was extremely heterogeneous in cancer diagnosis, (Table 5.1). Platelet hyperreactivity was observed across all patients and cancer types. This is not entirely surprising given that an increased incidence of thrombosis has been observed with many cancers [132]. The patients were was also heterogeneous in regard to medications used. Chemotherapy is known to further increase the risk of thrombosis in cancer patients [248]. However, only one patient enrolled in the study was undergoing chemotherapy at the time of platelet function testing. Hence chemotherapy regimens do not fully explain the increased platelet reactivity observed in this cohort. Furthermore, many of the patients were in fact on medications (aspirin, statins, nitrates, calcium channels inhibitors, ACE inhibitors and angiotensin receptor blockers, selective serotonin reuptake inhibitors, Table 5.1) that are known to inhibit platelet function to some degree [220, 249-254]. Thus, it can be concluded that the significant increase in platelet reactivity observed in patients with metastatic cancer is independent of the medications they were receiving.

Since it is difficult to recruit disease free, age matched, healthy controls when studying platelet function in older disease cohorts, the patients in this study are older than the healthy control population. It is well known that the incidence of arterial and venous thrombosis increases with age [255, 256]. Whether platelet reactivity increases with age is debatable and subject to conflicting reports. Terres *et al* reported a correlation between ADP and collagen induced platelet aggregation and increasing age[257]. However, in a much larger study, O'Donnell *et al* reported an

inverse correlation between ADP and epinephrine induced platelet aggregation and age, and no correlation between collagen induced platelet aggregation and age[258]. Regardless, a limitation of this study is the absence of age matched controls.

C-reactive protein (CRP) levels were significantly elevated in patients with metastaic cancer (27 ± 7 mg/l compared to the normal range of 0-5 mg/l, Table.5.1). CRP is a marker of inflammation and is elevated in association with many disease states, including atherosclerosis and cancer. Elevated CRP levels are associated with an increased incidence of arterial thrombosis (myocardial infarction and ischemic stroke)[259-261], but are not associated with an increased incidence of venous thrombosis [262, 263]. *In vitro*, heat aggregated CRP has been shown to act synergistically with ADP to increase platelet aggregation in washed platelet suspensions[264]. CRP has also been shown to increase platelet activation (platelet-monocyte aggregation) *in vitro*[265]. However, in that study, the ability of CRP to induce platelet-monocyte aggregation was highly dependent on the extracellular calcium ion level, and only occurred in heparinised platelet rich plasma, but not citrated platelet rich plasma. Since the study described in this chapter used sodium citrate as anticoagulant, it seems unlikely that elevated CRP levels were responsible for the increased platelet reactivity observed in the cohort of metastatic cancer patients.

In conclusion, it is demonstrated that patients with metastatic display global platelet hyperreactivity, as evidenced by increased spontaneous aggregation, increased agonist induced platelet aggregation, and increased agonist induced platelet activation. Since platelet hyperreactivity is associated with an increased incidence of both venous and arterial thrombosis, it seems highly likely that that this observation of platelet hyperreactivity could contribute to an increased incidence of thrombosis. Clinical trials of antiplatelet agents in conjunction with standard therapy using anticoagulants could be beneficial in the prevention of cancer associated thrombosis. Encouragingly, the results of the recently published WARFASA trial indicate that the addition of aspirin to standard warfarin therapy reduces the incidence of recurrent venous thrombosis [236].

Chapter 6: Conclusion and Future Work

Beyond their role in haemostasis, platelets are extremely important in the pathology of cancer. They are implicated in the promotion of growth and angiogenesis in the primary tumour[179], maintaining the integrity of tumour associated vasculature[95], and facilitating metastasis [56, 58, 60, 64, 68]. In this thesis, novel platelet tumour cell interactions that could potentially influence the pathology of cancer have been investigated, ranging from platelet induced signalling in ovarian cancer cells to platelet reactivity in patients with malignant disease.

In chapter 3, the effect of platelet adhesion and degranulation on signalling in tumour cells was explored. Platelet – tumour cell interactions have been shown to be critical for metastasis. For example, platelet adhesion to tumour cells (or 'platelet cloaking') and tumour cell induced platelet degranulation (TGFβ release) inhibit NK cell activity [59, 79, 85, 86, 89]. However, whether platelet adhesion and degranulation induce direct signalling events in tumour cells that could influence disease progression was previously not known. Conversely, in the case of atherosclerosis, it is well established that platelet induced signalling events can influence disease progression[159-162]. Based on this, it was hypothesised that platelet interactions with tumour cells could also induce signalling events in tumour cells that could potentially influence the progression of cancer. Using ovarian cancer cell lines as a model system, it was first demonstrated that ovarian cancer cell lines interact with platelets. A range of ovarian cancer cell lines were shown to support platelet adhesion and induce platelet activation and degranulation *(P-selectin expression/αIIbβ3 activation)*.

Many tumour cell lines have been shown to possess procoagulant activity [17, 157] which could induce activation of the coagulation cascade with subsequent platelet activation. In this study, platelet activation assays were performed in plasma based samples. It is possible that ovarian cancer cell induced platelet activation was a consequence of the activation of the coagulation cascade, leading to thrombin

generation, and thrombin induced platelet activation. However, hirudin (a potent thrombin inhibitor) had no effect on ovarian cancer cell induced platelet activation (Figure 3.5), suggesting that ovarian cancer cell induced platelet activation was independent of thrombin generation. Rather, platelet activation was shown to be dependent on the release of a soluble factor by ovarian cancer cells. This factor induced platelet activation via an ADP and P2Y1/P2Y12 receptor dependent mechanism (Figure 3.4). Preliminary results suggest ovarian cancer cells release ADP in a concentration insufficient to induce the extent of platelet activation observed (< 1μM in the supernatant of 1 x 10^6 59M cells/ml, data not shown). Hence, it is most likely that the unknown factor induces platelet activation that is dependent on the release of ADP from platelet dense granule stores. Ovarian cancer cell induced dense granule release could be easily quantified using the ATP/ADP release assay described in *chapter 2.22* and proteomic analysis could further determine the presence of platelet activating factors in the supernatant of ovarian cancer cells.

When it was established that ovarian cancer cells support platelet adhesion and induce platelet degranulation, the effect of platelet adhesion and degranulation on signalling events in ovarian cancer cells was investigated. Cells were treated with washed platelets *(representing platelet adhesion)* or platelet releasate *(representing platelet degranulatio*n) and gene expression profiling was performed. In response to washed platelets and platelet releasate, there was an increase (≥ 1.5 fold) in the expression of proliferative, anti-apoptotic, and pro-angiogenic genes in ovarian cancer cells. This was verified using both Affymetrix and Fluidigm gene expression arrays (Table 1.1). For example, the expression of the gene encoding for CCL2 was upregulated in 59M ovarian cancer cells following treatment with platelets or platelet releasate. This genes encodes for MCP-1 (monocyte chemoattractant protein-1), which has been shown to have proliferative, anti-apoptotic, and pro-angiogenic properties. A limitation of this study is that platelet induced signalling was demonstrated at the transcriptome level, but was not shown to lead to a measurable increase in protein synthesis. This could be easily assessed

in future studies by quantifying MCP-1 levels in 59M cells following treatment with platelets or platelet releasate.

While the results of this chapter represent *in vitro* data, platelet induced signalling in tumour cells has now been shown to be relevant *in vivo*. Labelle *et al* recently demonstrated that pretreating Ep5 breast cancer cells with platelets increased experimental metastasis in a murine model. Furthermore, they demonstrated that platelet derived TGFβ signalling activated the NFκB pathway ion Ep5 cells, leading to the increased expression of a number of pro-metastatic genes, notably CCL2. Inhibition of either platelet TGFβ or the NFκB pathway in Ep5 cells attenuated the pro-metastatic effect of platelets. Hence, platelet derived TGFβ induced signalling in Ep5 cells was responsible for potentiating metastasis; conclusively showing that platelet mediated signalling in tumour cells can influence disease progression in vivo[90].

More specifically with relation to ovarian cancer, the results presented in chapter 3 are strongly supported by the recent work of Stone *et al* [179]. The authors demonstrated that platelets infiltrate the primary tumour and promote growth and angiogenesis in a murine model of ovarian cancer. In the model, the induction of a 50 % reduction in platelet count coincided with a 50 % reduction in growth and angiogenesis within the primary tumour. This would suggest that platelets do induce proliferative, anti-apoptotic, and pro-angiogenic signalling in ovarian cancer cells *in vivo*. Interestingly, Cho et al have now demonstrated that platelets promote the proliferation of ovarian cancer cells (*in vitro)* via a TGFβ dependent mechanism. Furthermore, they showed that ovarian cancer patients with thrombocytosis display increased tumour proliferation indices compared to patients with normal platelet counts[266]. It is tempting to hypothesise that the increased gene expression in ovarian cancer cells observed in the study presented in chapter 3 is the result of TGFβ release by platelets and that platelet induced proliferation in the study of Cho *et al* was due to increased CCL2/MCP-1 synthesis. Consistent with this hypothesis, Labelle et al have convincingly shown that TGFβ signalling leads to increased MCP-1

synthesis[90]. This hypothesis could be investigated in the future by assessing the effect of CCL2 receptor blocking antibodies on proliferation in ovarian cancer cells.

In Chapter 4, platelets were shown to protect tumour cells in an *in vitro* model of shear induced damage. It has been widely suggested that platelets facilitate metastasis, in part, through their ability to protect tumour cells from shear induced damage[74, 90, 163]. Previously, there has been no evidence to support this hypothesis. Hence, the results of this study are the first evidence supporting the hypothesis that platelets can protect circulating tumour cells from shear induced damage.

This study was clearly limited by the use of only one tumour cell line and one marker of shear induced damage. The A2780 cell line was initially chosen since it supports platelet adhesion under shear conditions but fails to induce significant platelet activation even at high concentrations (Figure 3.2 & 3.3). Many tumour cell lines induce platelet activation *in vitro*, often only at supraphysiological concentrations. This can occur through the release of soluble platelet activating factors, resulting in the activation of both tumour cell adherent platelets as well as non adherent platelets. In patients, the concentration of circulating tumour cells is extremely low. As such, platelets would not encounter concentrations of tumour cells capable of inducing systemic platelet activation. By using the A2780 cell line, it was possible to exclude the effect of platelet degranulation, instead focusing on the direct effect of platelet cloaking (platelet – tumour cell adhesion) on shear induced damage in tumour cells. Using the A2780 cell line, platelet cloaking specifically, was shown to decrease membrane damage under shear conditions, most significantly at arterial shear rates (Figure 4.6). Under static or dynamic conditions, A2780 cells do not support high levels of platelet adhesion. The estimates of platelet adhesion suggested that less than 10 platelets adhered per A2780 cell, even in the presence of high concentrations of platelets (Figure 4.2). It could be hypothesised that with an increase in platelet cloaking (i.e.) increased numbers of adherent platelets, there would be a corresponding increase in the extent of protection from shear induced damage. This could be assessed in future studies using cell lines that support more platelet adhesion than the A2780 cell line (e.g.) the 59M cell line. Future studies

should also include more markers of shear induced damage (propidium iodide staining, annexin V staining, and caspase 3 activity) to verify the significance of LDH release.

In chapter 5, the results from a platelet function study performed in patients with advanced stage metastatic cancer were presented. In the study, patients with metastatic cancer were shown to display global platelet hyperreactivity, as evidenced by increased agonist induced platelet aggregation increased spontaneous platelet aggregation, and increased agonist induced platelet activation. *Ex vivo* studies have shown that platelet hyperreactivity is associated with an increased incidence of venous and arterial thrombosis. Hence, platelet hyperreactivity in this cohort could contribute to an increased incidence of cancer associated thrombosis.

While the results of this study are interesting, there are various limitations that could be addressed in the future. Platelet function testing was performed in plasma based samples. It is well known that patients with cancer show abnormalities in the coagulation cascade, for example, increased levels of coagulation proteins. As such, there is a possibility that the observed platelet hyperreactivity reactivity in this cohort is attributable to increased coagulation activation, leading to increased thrombin generation, and hence, increased platelet activation/aggregation. The hypothesis that platelet hyperreactivity in patients with metastaic cancer is due to increased coagulation activation could be easily assessed by performing the same platelet function study using washed platelet samples (free of coagulation factors) or plasma based samples pretreated with a thrombin inhibitor (PPACK or hirudin).

Platelet hyperreactivity is a multifactorial. Many other factors have been associated with increased platelet reactivity, some of which are clinically relevant to patients with cancer [238, 267-270]. For example, increased fibrinogen levels are associated with both increased agonist induced platelet aggregation and increased platelet P-selectin expression [258, 268]. Fibrinogen levels are commonly elevated in patients with cancer and are associated with poor prognosis. Fibrinogen is an acute phase protein

and hence increases during inflammation [271, 272]. Clearly, there is evidence of inflammation in this cohort of patients with metastatic cancer, as indicated by elevated C-reactive protein levels (Table 5.1). As such, platelet hyperreactivity in this cohort could be attributable to increased fibrinogen levels.

Another limitation of this study is the absence of an age matched control population. Although there is conflicting reports regarding the effect of age on platelet reactivity[257, 258], it would be more accurate to recruit disease free, aged matched controls when comparing platelet reactivity in healthy and disease populations. All 13 patients had a diagnosis of advanced untreatable metastatic cancer. This cohort was chosen since 90% of all patients with metastatic cancer show abnormalities in haemostasis[167]. Hence, it was likely that platelet hyperreactivity would be more evident in patients with metastatic cancer than patients with localised cancer. This hypothesis would also suggest that platelet reactivity would increase in disease progression, which could be assessed in future studies by comparing platelet reactivity in matched patients with localised and metastatic cancer.

In summary, this thesis demonstrates novel ways in which platelet function can influence malignancy and how in turn, malignancy can influence platelet function. Firstly, platelet adhesion and degranulation were shown to induce proliferative, anti-apoptotic, and pro-angiogenic signalling in ovarian cancer cells that could influence their metastatic potential *in vivo*. Secondly, platelets were shown to be capable of protecting cancer cells from shear stress induced damage. This could promote their survival within the circulation during bloodborne metastasis. Thirdly, patients with metastatic cancer were shown to display global platelet hyperreactivity, a phenomenon that could contribute to the high incidence of cancer associated thrombosis. This work adds to the growing body of literature demonstrating that platelets play a multifaceted role in the pathology of cancer. Given their importance, there is an increasingly compelling rationale to directly target platelet-tumour cell interactions as a therapeutic strategy for the treatment

of cancer. This warrants further investigation in randomised clinical trials of antiplatelet agents in the setting of cancer.

Appendix

7.0 Platelet function in a patient with a bleeding disorder of unknown cause

In an unrelated study, platelet function testing was performed on a patient with a bleeding disorder of unknown origin. With significance to chapter 5, these results also demonstrate that the assay used can detect decreased platelet reactivity, as well as increased platelet reactivity.

7.1 Introduction

The primary role of platelets is in haemostasis, the maintenance of vasculature integrity following vascular damage. Upon vascular damage, platelets rapidly adhere to exposed extracellular matrix proteins, become activated, then aggregate to form a haemostatic plug that prevents further blood loss (Figure 1.1). While increased platelet reactivity can be associated with thrombosis (Chapter 5.3), unsurprisingly, defects in platelet function are associated with a bleeding diathesis. Platelet function disorders are generally characterised by mild to moderate bleeding. This bleeding phenotype can range from easy bruising to prolonged episodes of bleeding following trauma or surgery. Muscle haematomas and haemarthroses (bleeding into joints) tend to be more rare in platelet function disorders and are more commonly associated with defects in secondary haemostasis, the propagation of coagulation reactions [273]. Platelet function disorders are extremely heterogeneous in nature and can be due to defects in platelet number, platelet adhesion, platelet aggregation, secretion, and intracellular signalling [274-277]. Due to their complexity, the diagnosis of platelet function disorders is often difficult and is further hampered by the lack of standardised testing methods [278, 279].

The National Centre for Heritable Coagulation Disorders (NCHCD) at St James' Hospital deals with patients with platelet function disorders. They contacted our lab

regarding a patient, a 38 year old male with a lifelong bleeding tendency. The patient has a history of prolonged bleeds following minor trauma. In the past, the patient has also developed deep muscle haematomas and haemarthroses following minor trauma. In childhood, he was diagnosed with a platelet function disorder based on a reversible aggregation to ADP and a slightly increased bleeding time. After a number of years away from the clinic, he recently represented at the NCHCD with a large haematoma in his right thigh causing compression of the peroneal nerve with foot drop.

The first wave of testing carried out at the NCHCD revealed the patient had a normal prothrombin time, normal activated partial thromboplastin (time, normal vWF, fibrinogen, factor FXII, and intrinsic and extrinsic coagulation factor levels. Platelet count and platelet size were within the normal range. The patient had normal levels of platelet nucleotides (ADP and ATP levels). Arachidonic acid and ristocetin induced platelet aggregation was normal. Platelet aggregation responses to collagen and epinephrine were reduced on one occasion, normal on another occasion. In fact, the only consistent result indicating decreased haemostatic function was a decreased aggregation response to ADP, an abnormality that does not fully explain the severity of bleeding. When contacted by the NCHCD, we were requested to perform assays of phosphatidylserine exposure and ATP release to rule out Scotts Syndrome (an abnormality in membrane phospholipids expression) and a dense granule release defect, respectively.

Platelet function testing on the NCHCD patient was carried out at the Royal College of Surgeons in Ireland on two separate occasions, separated by 2 months. On each occasion, platelet function in a healthy control was carried out in parallel. We tested four parameters of platelet function. These were platelet aggregation (by *aggregometry, n = 2*), dense granule release (*by ATP release, n = 1*), alpha granule release (*by P-selectin expression, n = 2*), and phosphatidylserine exposure (*annexin v binding, n = 1*). The NCHCD patient displayed significantly decreased platelet aggregation response to ADP and epinephrine, slightly decreased phosphatidylserine exposure in response to multiple agonists, and significantly decreased alpha granule

release in response to multiple agonists. The significance of these results and how they may contribute to the bleeding phenotype observed in this patient are detailed in the discussion section (section 7.3).

7.2 Results

7.2.1 Decreased ADP and epinephrine induced platelet aggregation in the NCHCD patient

To assess platelet aggregation, aggregation responses to arachidonic acid, collagen, ADP, epinephrine, and PAR-1 activating peptide in the NCHCD patient were assayed using the previously described 96 well plate modification of light transmission aggregometry. Platelet aggregation responses were measured on two separate occasions. Consistent with results from the NCHCD, the patient displayed a significantly decreased aggregation response to ADP compared to healthy controls ($n = 10$). For example, in response to 2.5 μM ADP, platelet aggregation was 14 ± 8 % in the NCHCD patient compared to 51 ± 5 % in healthy controls ($n = 10$, $p \leq 0.05$). Platelet aggregation responses to epinephrine were also significantly decreased in the NCHCD patient. For example, in response to 1.25 μM epinephrine, platelet aggregation was 6 ± 6 % in the NCHCD patient compared to 47 ± 5 % in healthy controls ($n = 10$, $p \leq 0.05$). There was no significant difference in platelet aggregation responses to arachidonic acid, collagen and PAR-1 activating peptide between the patient and healthy controls (Figure 7.1, 7.2 and 7.5).

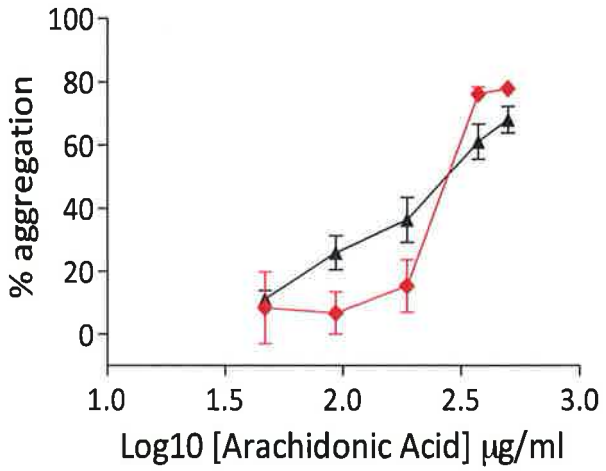

Figure 7.1: Arachidonic acid induced platelet aggregation responses are similar in the NCHCD patient and healthy controls (mean + SEM). Platelet aggregation responses to multiple concentrations of arachidonic acid were measured in the **NCHCD patient (n = 2)** and compared to **healthy controls (n = 10).** Arachidonic acid is converted via prostaglandin synthesis to thromboxane A2, which induces platelet activation and aggregation via the TxA2 (TP) receptor. There was no difference between arachidonic acid induced aggregation responses in the NCHCD patient and healthy controls. Data was analysed using a student t test.

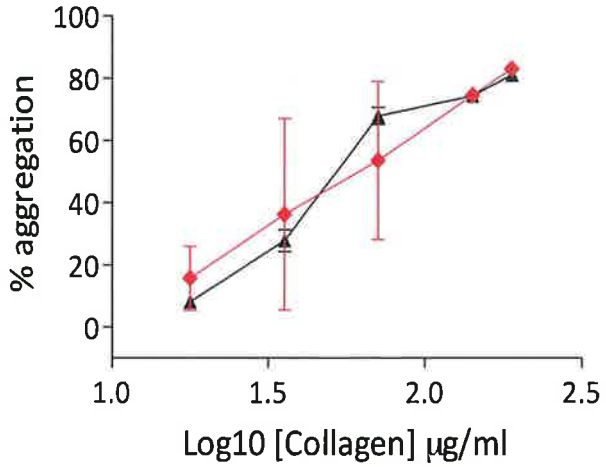

Figure 7.2: Collagen induced platelet aggregation responses are similar in the NCHCD patient and healthy controls (mean + SEM). Platelet aggregation responses to multiple concentrations of collagen were measured in the **NCHCD patient (n = 2)** and compared to **healthy controls (n = 10).** Collagen induces platelet activation and aggregation via GPVI and α2β1. There was no significant difference between collagen induced aggregation responses in the NCHCD patient and healthy controls. Data was analysed using a student t test.

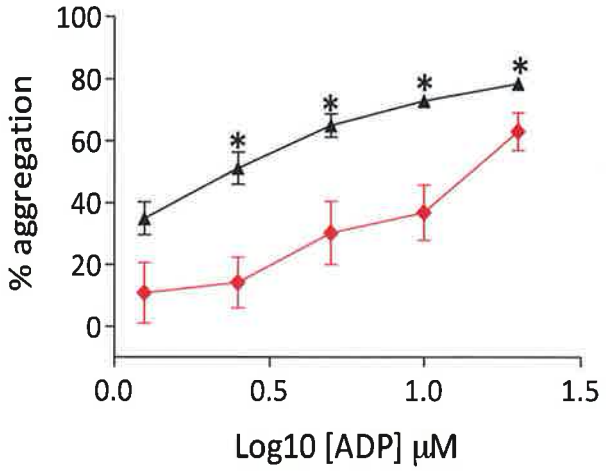

Figure 6.3: Decreased ADP induced platelet aggregation in the NCHCD patient (mean + SEM). Platelet aggregation responses to multiple concentrations of ADP were measured in the **NCHCD patient (n = 2)** and compared to **healthy controls (n = 10).** ADP induces platelet activation via the purinergic receptors P2Y1 and P2Y12. Platelet aggregation was significantly decreased in the NCHCD patient in response to 2.5, 5, 10 and 20 µM ADP ($p \leq 0.05$). Data was analysed using a student t test.

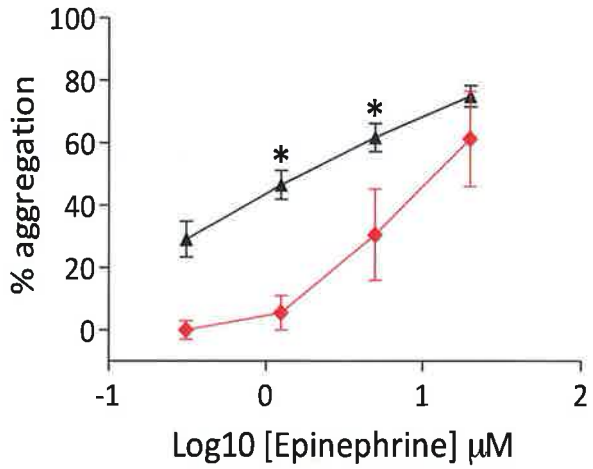

Figure 6.4: Decreased epinephrine induced platelet aggregation in the NCHCD patient (mean + SEM). Platelet aggregation responses to multiple concentrations of epinephrine were measured in the **NCHCD patient (n = 2)** and compared to **healthy controls (n = 10).** Epinephrine induces platelet activation and aggregation via the α2 adrenergic receptor. Platelet aggregation was significantly decreased in the NCHCD patient in response to 1.25 and 5 µM epinephrine ($p \leq 0.05$). Data was analysed using a student t test.

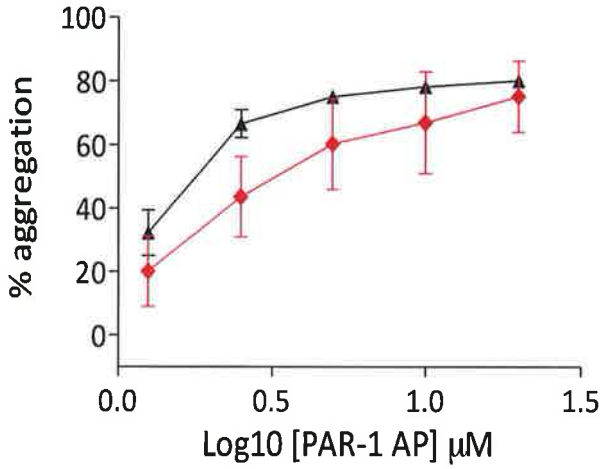

Figure 7.5: PAR-1 activating peptide induced platelet aggregation is slightly decreased in the NCHCD patient (mean + SEM). Platelet aggregation responses to multiple concentrations of PAR-1 activating peptide were measured in the **NCHCD patient (n = 2)** and compared to **healthy controls (n = 10).** PAR-1 activating peptide induces platelet activation and aggregation via the PAR-1 receptor. % platelet aggregation in response to PAR-1 activating peptide was slightly but not significantly decreased in the NCHCD patient. Data was analysed using a student t test.

7.2.2 Normal dense granule release in the NCHCD patient

To assess dense granule release in the NCHCD patient, agonist induced ATP release was assayed using a 96 well plate luminescence based assay. This assay utilises the ATP dependent luciferin - luciferase reaction, a reaction that generates a luminescent light signal that is proportional to the amount of ATP present in a sample. Results are expressed as arbitrary luminescence units (ALU). In response to multiple concentrations of PAR-1 activating peptide, PAR-4 activating peptide, and collagen, ATP release in the NCHCD patient was comparable to healthy controls, suggesting that the patient does not suffer from dense granule secretion defect (Figure 7.6). For example, ATP release in response to 20μM PAR-1 activating was 213000 ± 17000 (n = 5) ALU in healthy controls compared to 240000 ALU in the NCHCD patient (n = 1). In response to 150 μM PAR-4 activating peptide, ATP release was 180000 ± 24000 ALU in healthy controls (n = 4) compared to 180000 ALU in the NCHCD patient. In response to 71.25 μg/ml collagen, ATP release was 200000 ALU in the NCHCD patient compared to 200000 ± 16000 ALU in healthy controls (n = 5).

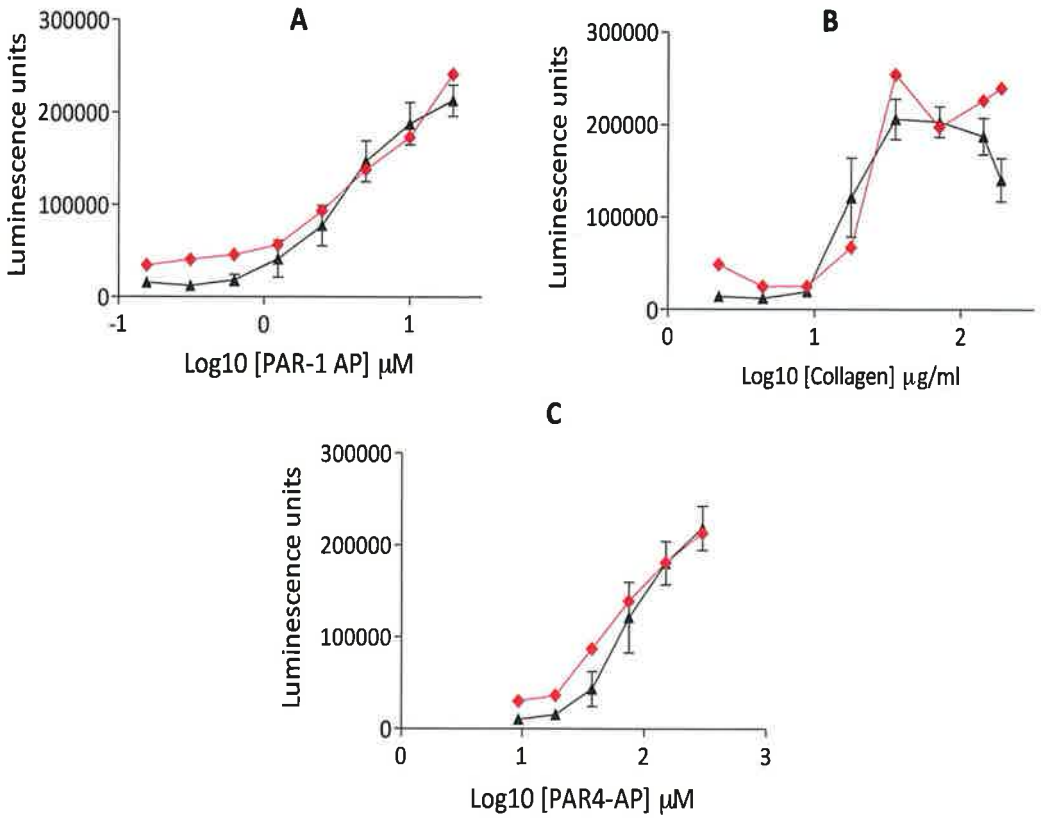

Figure 7.6: Platelet dense granule release is similar in the NCHCD patient and healthy controls (mean ± SEM). Dense granule release was assessed by platelet ATP release using an assay based on the ATP dependent luciferin-luciferase reaction. Results are expressed as arbitrary luminescence units. In response to multiple concentrations of PAR-1 activating peptide (A), PAR-4 activating peptide (B), and collagen (C), ATP release in the **NCHCD patient (n = 1)** was comparable to **healthy controls (n = 4 - 5)**, suggesting that the patient does not suffer from dense granule secretion defect..

7.2.3 Decreased agonist induced phosphatidylserine exposure in the NCHCD patient

To assess phosphatidylserine exposure in the NCHCD patient, agonist induced phosphatidylserine exposure was measured by fluorescently labelled annexin V binding. Results are expressed as the percentage of platelets positive for annexin V binding relative to resting unactivated platelets. In response to PAR-1 activating peptide (20 μM), PAR-4 activating peptide (300 μM), Convulxin (a GPVI agonist, 50 ng/ml), and all 3 agonists in combination, annexin V binding was slightly reduced in the NCHCD patient (n = 1) compared to healthy controls (5-7) (Figure 7.7). For example, in response to 50 ng/ml convulxin, the percentage of platelets positive for annexin V binding was 17 % in the NCHCD patient compared to 41 ± 6 % in healthy controls. In response to PAR-1 activating peptide, PAR-4 activating peptide (300 μM), and convulxin in combination, the percentage of platelets positive for annexin v binding was 47 % (n = 1) compared to 70 ± 4 in healthy controls (n = 7).

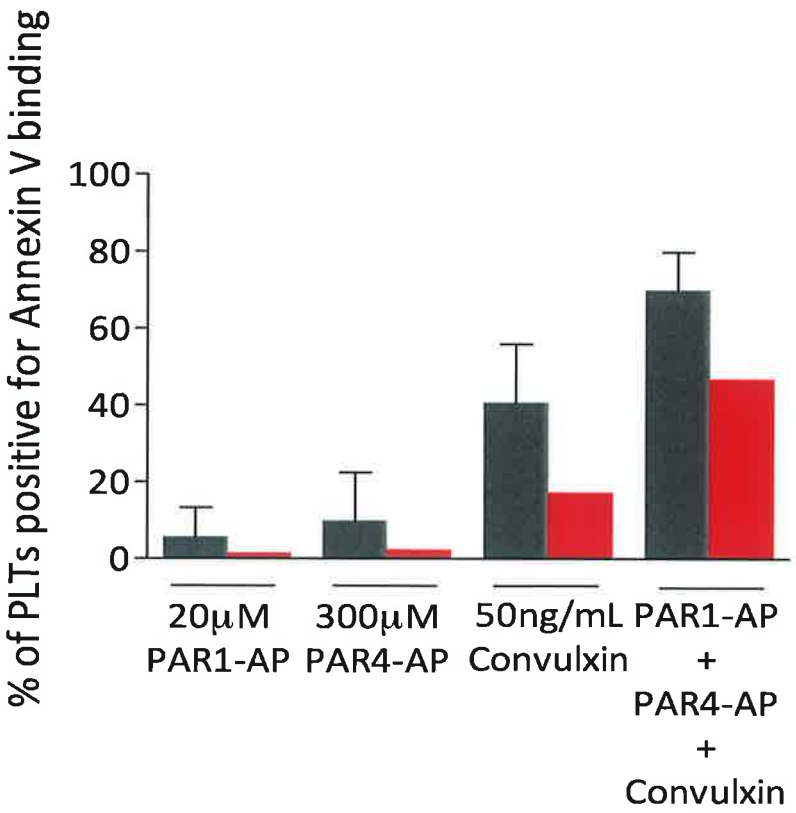

Figure 7.7: Phosphatidylserine exposure is slightly decreased in the NCHCD patient compared to healthy controls (mean + SEM). Phosphatidylserine exposure in the NCHCD patient was assessed by annexin V binding. In response PAR-1 activating peptide (20 μM), PAR-4 activating peptide (300 μM), Convulxin (50 ng/ml), and all 3 agonists in combination, annexin V binding was slightly decreased in the **NCHCD patient (n = 1)** compared to **healthy controls (5 -7)**.

7.2.4 Decreased alpha granule release in the NCHCD patient

To assess platelet α-granule release in the NCHCD patient, agonist induced P-selectin expression was assayed. In contrast to the assay described in chapter 5 that used whole blood samples, agonist induced P-selectin was measured in PRP samples. Results are expressed as the percentage of platelets P-selectin positive relative to an isotype control. Agonist induced P-selectin expression was significantly decreased in the NCHCD patients in response to collagen, ADP, and PAR-1 activating peptide, even at high concentrations (Figure 7.8 a – c), suggesting the patient has a defect in either α-granule storage or release. For example, % P-selectin expression was decreased in the NCHCD patient (n = 2) compared to healthy controls (n = 3-6) in response to 190 μg/ml collagen (17 ± 17 % vs. 60 ± 14, $p \leq 0.05$), 10 μM ADP (13 ± 13 % vs. 61 ± 15, $p \leq 0.05$), and 10 μM PAR-1 activating peptide (55 ± 17 %, vs. 91 ± 7, $p \leq 0.05$). This decrease in P-selectin expression was also seen at concentrations of collagen (190 μg/ml) and PAR-1 activating peptide (20 μM) that consistently induced platelet aggregation responses comparable to healthy controls (Figure 7.2 and 7.5).

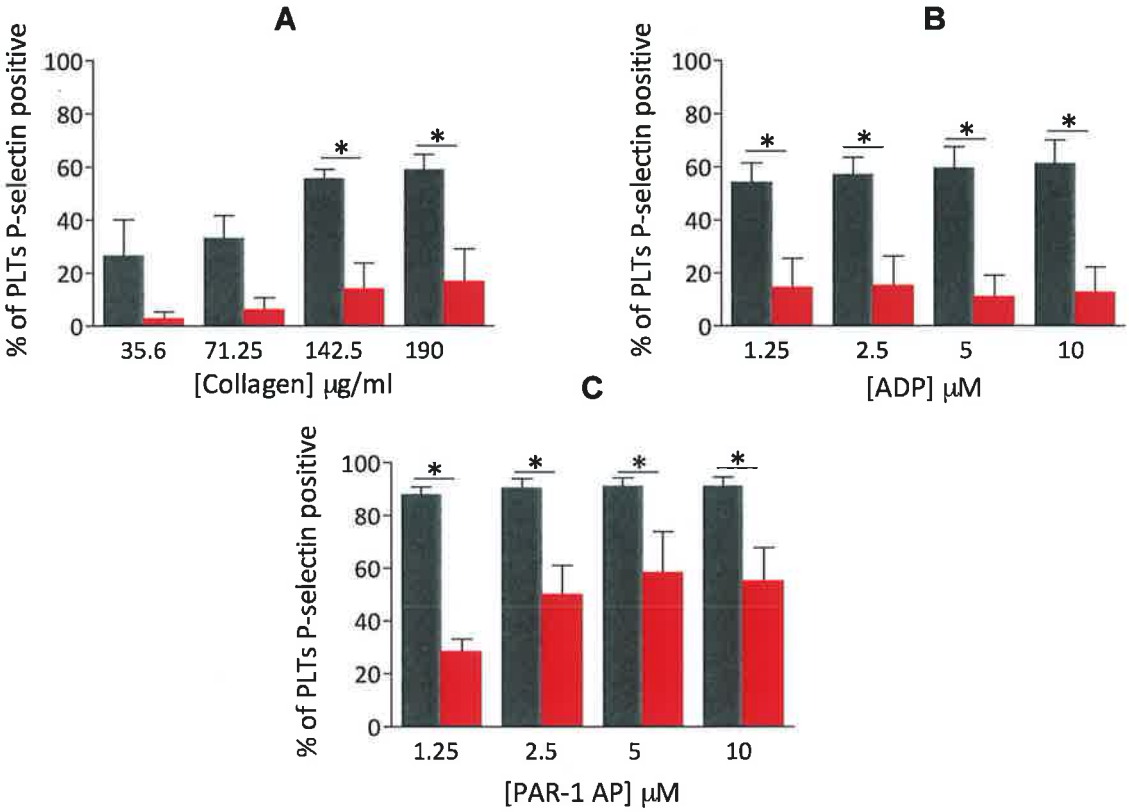

Figure 7.8: Platelet α granule release is significantly decreased in the NCHCD patient (mean + SEM). α-Granule release in the NCHCD patient was assessed by P-selectin expression. In response to multiple concentrations of collagen **(A)**, ADP **(B)**, and PAR-1 activating peptide **(C)**, agonist induced P-selectin expression was significantly decreased in the NCHCD patient (n = 2) compared to **healthy controls (n = 3 – 6,** * = $p \leq 0.05$). This decrease in P-selectin expression was also evident at concentrations of PAR-1 activating peptide and collagen that induced platelet aggregation comparable to healthy controls (Figure 7.2 and 7.5). Data was analysed using a student d t test.

7.3 Discussion

In this case study, a patient with a bleeding disorder of unknown cause was tested for abnormalities in platelet function. In the first wave of testing at the National Centre for Heritable coagulation Disorders, St James' Hospital, an abnormality in ADP induced platelet aggregation was identified. Consistent with the findings at the NCHCD, we found a markedly decreased aggregation response to ADP, even at high concentrations (Figure 7.3). ADP is a relatively weak but important platelet agonist, stored in and secreted from platelet dense granules upon activation. It serves to amplify platelet aggregation in response to other agonists [115-117]. In platelets, P2Y1 elicits shape change, Ca^{2+} mobilisation, and rapid reversible platelet aggregation, while P2Y12 elicits slowly progressive platelet aggregation without shape change[280]. Simultaneous activation of both receptors by ADP is required for normal platelet aggregation and thrombus formation *in vivo* [280]. As such, both P2Y1 and P2Y12 deficient mice are protected against experimental thrombosis [281-283].

The absence of a defect in dense granule storage (normal ADP/ATP levels) or dense granule release (Figure 7.6) and the fact that the decreased aggregation response to ADP was still evident in response to high concentrations of ADP (Figure 7.3) suggests that there may be a defect in P2Y1 or P2Y12 expression or signalling. Cases of congenital defects in both the P2Y1 and P2Y12 receptor have been reported in the literature. These defects are associated with a mild bleeding phenotype [281, 282]. Oury *et al* reported a patient with a defect in the transcription of the P2Y1 receptor gene. The patient had a normal P2Y1 gene but decreased platelet levels of P2Y1 mRNA. Consistent with a P2Y1 deficiency, the patient displayed weak platelet aggregation and no calcium mobilisation in response to ADP[284]. Six cases of congenital defects in P2Y12 have been described in the literature, resulting from mutations in the P2Y12 gene. Patients with a P2Y12 deficiency are characterised by a prolonged bleeding time (15-20 minutes), reversible platelet aggregation to weak agonists, and the complete absence of platelet aggregation in response to high concentrations of ADP (> 10 μM) [281, 282, 285, 286]. Whether decreased ADP induced aggregation in the NCHCD patient represents a defect in P2Y1 or P2Y12 receptor

transcription, expression, or signalling following ADP binding remains to be determined.

Due to the amplifying effect of ADP on platelet aggregation, defects in responses to ADP can also present as abnormalities in platelet aggregation responses to other agonists. This may explain the decreased aggregation responses to epinephrine, and the variable aggregation responses to intermediate concentrations of collagen (Figure 7.2 & 7.4). As agonist concentrations decrease, platelet aggregation becomes more and more dependent on the amplifying effect of ADP release from dense granules. However, defects in ADP induced signalling do not explain decreased platelet P-selectin expression in response to PAR-1 activating peptide and collagen. Inhibition of the P2Y1 receptor, P2Y12 receptor or ADP is not associated with decreased P-selectin expression in response to high concentrations of PAR-1 activating peptide in healthy controls (Figure 3.4c). The decreased P-selectin expression in the NCHCD patient suggests a defect related to platelet alpha granules.

P-selectin is a membrane bound adhesion molecule present on the surface of platelet α granules and the Weibel Palade bodies of endothelial cells. Upon platelet activation, α granules fuse with the platelet plasma membrane and release their contents into the extracellular space. During this process, P-selectin is translocated to the surface of the platelet, and hence, it serves as a marker of platelet α-granule release. While the role of P-selectin in inflammation is well known, more recently its role in haemostasis has been appreciated. P-selectin mediates the accumulation of tissue factor bearing microparticles in a growing thrombus that ultimately facilitate tissue factor dependent fibrin formation. The generation of a fibrin meshwork serves to stabilise the growing thrombus [287, 288]. As such, P-selectin deficient mice form thinner and less compact thrombi than wild type mice [289, 290]. Hence, decreased expression of P-selectin expression in the NCHCD patient could result in reduced fibrin formation *in vivo,* ultimately leading to reduced thrombus stability. As of yet, a congenital defect in P-selectin has not been described in the literature. Furthermore, while the NCHCD patient clearly displays decreased P-

selectin expression, whether this is due to impaired α-granule release or storage needs to be determined.

α-Granules are the main storage granule in platelets, with a approximately 50 - 80 per platelet, ranging in size from 200 – 500 nm. They contain an array of proteins including membrane bound receptors (GPVI, β3, αIIb, GP-Ib-IX-V complex), coagulation factors (Factors V, XI, XIII), and adhesive proteins (vWF, fibrinogen). These proteins are packaged into α granule during megakaryopoiesis or via receptor mediated endocytosis during platelet circulation. Congenital defects in platelet alpha granules have been reported, most notably Gray Platelet Syndrome (GPS) and Quebec Platelet Disorder [291-293].

GPS is an autosomal recessive α-granule disorder characterised by mild bleeding. The disorder is so called because the inability of platelets to store α-granule proteins causes them to appear gray on May-Grunwald-Giesma stained blood smears. A common feature of GPS is the early onset of myelofibrosis (the replacement of bone marrow by scar tissue), due to the inability of megakaryocytes to store fibrogenic α-granular proteins. While reduced levels of P-selectin have been reported in association with GPS, giant platelets and thrombocytopenia are commonly seen with GPS, characteristics not seen in the NCHCD patient. The Quebec platelet disorder is an autosomal dominant α-granule disorder, first identified in two French Canadian families. It is associated with mild to moderate bleeding. It is caused by the aberrant expression and storage of the fibrinolytic enzyme urokinase-type plasminogen activator in platelet α-granules, leading to the degradation of platelet α-granule proteins. A hallmark of this disorder is late onset bleeding (up to 24 hours post trauma) that responds to fibrinolytic inhibitors but not platelet transfusions. Another hallmark of Quebec platelet disorder is the absence of epinephrine induced aggregation, the cause of which is unknown [291-293], again a feature not observed in the NCHCD patient.

When contacted by the NCHCD, testing for Scott Syndrome was requested. This is an extremely rare inherited bleeding disorder caused by the inability of platelets to

translocate phosphatidylserine to the outer leaflet of the plasma membrane upon activation. This stops factor Xa and Va binding to the surface of activated platelets, resulting in the inability of platelets to transform prothrombin to thrombin[294, 295]. The NCHCD patient displayed slightly decreased PS exposure (measured by annexin V binding) compared to healthy controls. This was on a single occasion and would require repeat testing to confirm. However, Scott Syndrome patients present with a mild bleeding phenotype in association with normal platelet aggregation and a normal bleeding time[292], characteristics that are not evident in the NCHCD patient who also shows abnormalities in platelet aggregation and α-granule release or storage.

In summary, abnormalities in platelet function were identified in a patient with a bleeding disorder of unknown cause. These abnormalities could contribute to, but do not fully explain the severity of bleeding in this patient. The decreased responses across multiple parameters of platelet function (aggregation, secretion, phosphatidylserine exposure) suggest a possible defect in intracellular signalling that requires further investigation. The task of identifying the root of this platelet function defect could be extremely difficult and require a significant amount of further testing. Also, the severity of bleeding seen in this patient (muscle haematomas and haemarthroses) is uncharacteristic of platelet function disorders, suggesting that the patient may display other haemostatic defects.

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